AWARD NUMBER:	W81XWH-04-1-0444
TITLE:	Pharmacological and behavioral enhancement of neuroplasticity in the MPTP-lesioned mouse and nonhuman primate.
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REPORT DATE:	May 2009
TYPE OF REPORT:	Final
PREPARED FOR:	US Army Medical Research and Material Command Fort Detrick, Maryland, 21702-5012
DISTRIBUTION STATEMENT:	Approved for Public Release Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. Agency Use Only (Leave blank)	2. Report Date	3. Report Type and Period Covered (i.e., annual 1 Jun 00 - 31 May 01)
	31-05-2009	Final MAY 2004 - 30 APR 2009

4. Title and Subtitle
Pharmacological and behavioral enhancement of neuroplasticity in the MPTP-lesioned mouse and nonhuman primate.

5. Award Number
W81XWH-04-1-0444

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8. Performing Organization Report Number (Leave Blank)

9. Sponsoring/Monitoring Agency Name and Address
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. Sponsoring/Monitoring Agency Report Number (Leave Blank)

11. Supplementary Notes (i.e., report contains color photos, report contains appendix in non-print form, etc.)
Report contains Appendices in electronic form.

12a. Distribution/Availability Statement (check one)
Approved for public release; distribution unlimited

12b. Distribution Code
(Leave Blank)

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The purpose of this proposal is to investigate the mechanisms involving pharmacological and behavioral enhanced neuroplasticity of the injured basal ganglia. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions. This proposal has two components. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the D2 receptor agonist (Pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
MPTP, neuroplasticity, models of injury, repair, basal ganglia

15. Number of Pages (including appendices) 181

16. Price Code (Leave Blank)

17. Security Classification of Report	18. Security Classification of this Page	19. Security Classification of Abstract	20. Limitation of Abstract
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

The following Report is a Final Annual Report assembled upon completion of year 5. Year 5 was in the status of a "no cost extension" to allow completion of the intended studies within the Specific Aims and publication of manuscripts that have emerged from this granting period. In addition we would like to point out that data from these studies supported through the NETRP have led to submission of several applications to the National Institutes of Health (listed in the Appendix) including a human clinical trial examining the benefits of exercise in patients with Parkinson's disease and a RO1 grant starting June 15, 2009 extending findings from these supported studies to further molecular analysis of the underlying mechanisms involved in enhanced recovery following basal ganglia injury in the MPTP-lesioned mouse model of Parkinson's disease.

For the purpose of this Final Report we have extended our report from Year 4 that included outcomes and highlights from all previous years. While this adds additional material to this report we felt it was beneficial to emphasize the progress of this grant and the achievements and any deviations from the original aims. This arrangement allows for evaluation of progress for Year 5 as well as evaluation of the entire 5-year period.

The primary focus of this research grant was to determine the underlying mechanisms responsible for neuroplasticity in the injured adult basal ganglia. For these studies we utilize the neurotoxicant MPTP that selectively destroys nigrostriatal dopaminergic neurons and leads to the depletion of striatal dopamine as well as the development of parkinsonian features. In the squirrel monkey these features include slowness of movement, balance impairment and diminished hand dexterity. In our laboratory we utilize both the MPTP-lesioned C57BL6 mouse and the MPTP-lesioned squirrel monkey. Both models show intrinsic plasticity through either striatal dopamine return (mouse) and/or behavioral recovery (squirrel monkey). In this proposal we were particularly interested in understanding whether exercise (mouse) or dopamine replacement therapy (monkey) might enhance intrinsic neuroplasticity of the injured basal ganglia. For this purpose, the proposal was divided into two components, a mouse exercise study and a squirrel monkey dopamine replacement study. These studies were designed to be complementary in that both non-pharmacological and pharmacological effects of neuroplasticity are being investigated.

In the following sections are included the abstract, introduction and specific aims from the original proposal. This is followed by the accomplishments and research outcomes from Year one through Year 5. This annual report also includes manuscripts in the form of appendices or their electronic links.

Abstract (From the Original Application)

The purpose of this proposal is to investigate the molecular mechanisms involving pharmacological and behavioral (exercise) enhanced neuroplasticity of the injured basal ganglia. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions. The following proposal has two complementary components using two animal models to address the molecular mechanisms underlying exercise- and pharmacologically-enhanced neuroplasticity. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be further tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the administration of a D2 receptor agonist (Pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses. By elucidating the role of exercise and pharmacological manipulation in neuroplasticity of the injured brain we hope to identify novel therapeutic

targets for the treatment of brain injury and neurotoxic insult. Since military personnel are at risk for a wide range of brain injuries including head trauma, neurotoxic exposure (from pesticides, hostile enemy poisoning, viral and biological weapon based agents) it is imperative that medical strategies be made available to reverse the debilitating neurological deficits.

D: STATEMENT OF WORK (From the original Application)

The brain's capacity for recovery from damage is far greater than previously recognized. It is now understood that neuroplasticity can be modulated through activity-dependent processes including exercise and environmental enrichment, and through pharmacological manipulation. Most of our understanding of exercise and pharmacological enhanced neuroplasticity is derived from studies in the cortex and the hippocampus, but there is mounting evidence that the same phenomenon occurs in the injured basal ganglia. The molecular mechanisms for this phenomenon are not well understood. Using two animal models of injury induced neuroplasticity in the basal ganglia (the MPTP-lesioned mouse and MPTP-lesioned non-human primate) we propose to examine two modes of intervention to enhance neuroplasticity. These include exercise in the MPTP-lesioned mouse model and pharmacological intervention in the MPTP-lesioned non-human primate. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions.

The following proposal has two complementary components using both animal models to address the molecular mechanisms underlying exercise- and pharmacologically-enhanced neuroplasticity. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be further tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the administration of a D2 receptor agonist (pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses. By elucidating the role of exercise and pharmacological manipulation in neuroplasticity of the injured brain we will identify new therapeutic targets for the treatment of traumatic brain injury and neurotoxic insult, two high-risk morbidities that are common to military personnel.

<u>Component One</u>: To test the hypothesis that exercise enhances neuroplasticity of the MPTP-lesioned mouse through glutamate by modulating dopamine biosynthesis.

Component One will utilize the following 4 treatment groups for **Study1** through **Study 4**:

(1) Saline-injected;
(2) MPTP-injected;
(3) Saline-injected + exercise;
(4) MPTP-injected + exercise.

Study 5 will utilize the following glutamate antagonists: AMPA antagonist (GYKI-52466) and the NMDA antagonist (MK-801) in the following 8 treatment groups:

(1) Saline-injected + GYKI-52466;
(2) MPTP-injected + GYKI-52466;
(3) Saline-injected + exercise + GYKI-52466;
(4) MPTP-injected + exercise + GYKI-52466;
(5) Saline-injected + MK801;
(6) MPTP-injected + MK801;
(7) Saline-injected + exercise + MK801;
(8) MPTP-injected + exercise +MK801.

Exercise will be performed on a motorized rodent treadmill. Brain tissue will be collected after 30 days of running.

Study 1: The level of striatal dopamine and its metabolites will be determined using HPLC analysis comparing exercise versus non-exercise groups in the MPTP-lesioned mouse.

Study 2: The pattern of expression of striatal tyrosine hydroxylase (TH), dopamine transporter (DAT), cAMP-responsive enhancer binding protein (CREB), phospho~CREB, and dopamine- and adenosine- 3':5'-monophosphate-regulated phosphoprotein (DARPP-32), and phospho~DARPP-32 protein and their mRNA transcripts in surviving dopaminergic neurons will be determined using immunohistochemistry, western immunoblotting, *in situ* hybridization and correlated with striatal dopamine return. Pilot data shows attenuation of the return of DAT protein, and TH mRNA by exercise in MPTP-lesioned mice.

Study 3: The effect of exercise on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows altered glutamatergic synapses using immuno-electron microscopy.

Study 4: The pattern of expression of subunits for both the NMDA and AMPA receptor subtypes and their phosphorylated state will be determined using western immunoblotting, immunocytochemistry and *in situ* hybridization histochemistry.

Study 5: *We will test the hypothesis that exercise induced neuroplasticity can be attenuated through the administration of either a NMDA or AMPA receptor antagonist*. After MPTP-lesioning mice will be subjected to exercise while receiving either the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist GYKI-52466. Behavioral recovery will be compared between groups. Brain tissue will be analyzed for alteration in dopaminergic function (dopamine, DAT and TH expression). Pilot studies show that both glutamate receptor antagonists GYKI-52466 and MK-801 can be administered in this model of MPTP-lesioning.

Component Two: To test the hypothesis that the administration of a D2 receptor agonist (pramipexole) enhances neuroplasticity of the MPTP-lesioned non-human primate through its effect on dopamine (biosynthesis, uptake, and receptor expression) and glutamatergic synapses.

Component Two will utilize the following treatment groups (n = 4 per group):

(1) Saline-injected harvested at 6 weeks after the last injection;
(2) Saline-injected harvested at 16 weeks after the last injection;
(3) MPTP-injected harvested at 6 weeks after the last injection;
(4) MPTP-injected harvested at 16 weeks after the last injection;
(5) Saline-injected + pramipexole harvested at 6 weeks after the last injection;
(6) Saline-injected + pramipexole harvested at 16 weeks after the last injection;
(7) MPTP-injected + pramipexole harvested at 6 weeks after the last injection;
(8) MPTP-injected + pramipexole harvested at 16 weeks after the last injection.

Study 1: The behavioral recovery of saline injected and MPTP-lesioned squirrel monkeys will be compared with and without the administration of pramipexole. Animal behavior will be monitored using both a cage side clinical rating scale and a personal activity monitor.

Study 2: The pattern of expression of proteins and mRNA transcripts important for dopaminergic function, (including TH, DAT, VMAT2) at the level of the SNpc and CPu will be determined. Preliminary data supports our ability to carry out western immunoblotting, immunocytochemistry and *in situ* hybridization in the MPTP-lesioned non-human primate.

Study 3: The pattern of expression of the dopamine receptors D1, D2, and D3 will be determined in both the SNpc and CPu. The level of protein expression will be determined western immunoblotting, immunohistochemistry, while the level of mRNA transcript expression will be determined using *in situ* hybridization histochemistry. Double labeling techniques will be used to co-localize the dopamine receptor changes with other enkephalin or substance P containing neurons. Preliminary data supports our ability to use these techniques in the non-human primate.

Study 4: The effect of pramipexole on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows our ability to quantify glutamatergic synapses using immuno-electron microscopy.

At the conclusion of these studies we will have a better understanding on the role of exercise and dopamine agonist (pramipexole) treatment in enhancing neuroplasticity of the injured basal ganglia in the mouse and the non-human primate. This may then identify important therapeutic targets (through glutamate and dopamine) for the treatment of brain injury.

Table 1: Timeline of Experimental Design for Component One (Exercise in the MPTP-Lesioned Mouse Model).

Component One: Exercise in the MPTP-lesioned Mouse				
	Year 1	**Year 2**	**Year 3**	**Year 4**
Study 1: Analysis of Dopamine and its metabolites	HPLC			
Study 2: Analysis of TH, DAT, CREB, and DARPP-32	Immunocytochemistry, In Situ Hybridization, Western Immunoblotting			
Study 3: Analysis of striatal glutamate synapses		Immuno-electron microscopy		
Study 4: Analysis of NMDA and AMPA receptor subtypes		Immunocytochemistry, In Situ Hybridization, Western Immunoblotting		
Study 5: Attenuate neuroplasticity with NMDA and AMPA receptor antagonists			Immunocytochemistry, In Situ Hybridization, Western Immunoblotting, Immuno-electron microscopy	

<u>Component 2</u>: <u>Pharmacological Enhancement of Neuroplasticity in the MPTP-lesioned Non-Human Primate Model.</u>

Time Line:

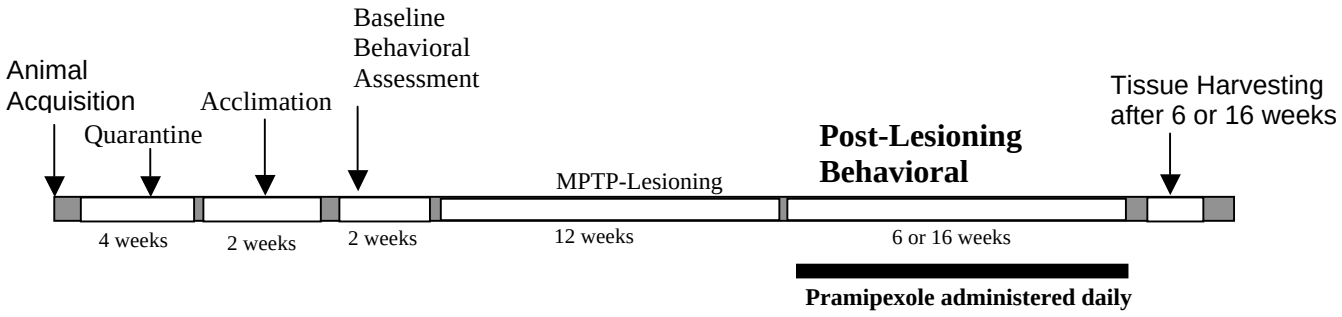


Table 2: Timeline of Experimental Design for Component Two (MPTP-Lesioned Primate Model).

Specific Aim	**Year 1**	**Year 2**	**Year 3**	**Year 4**
	Lesion animals and administer Pramipexole			
Study 1: Behavior	Behavioral analysis			
Study 2: Molecular		TH, DAT, VMAT mRNA and protein using WIB, ICC and ISH		
Study 3: Dopamine Receptors		Dopamine Receptor D1, D2, and D3 using WIB, ICC, and ISH		
Study 4: Glutamate		Analysis of glutamatergic synapses using immuno-EM		

Key Research Accomplishments for Years One Through Three

Component One: Enhancement of neuroplasticity in the MPTP-lesioned mouse

(i) Intensive treadmill exercise leads to improved motor performance of both MPTP-lesioned and saline treated mice. Specifically exercised animals run faster and for a longer duration. This is published in Fisher et al 2004 and Petzinger et al 2007.

(ii) Analysis of behavior using the accelerating rotarod as a second measure shows that both saline and MPTP-lesioned mice demonstrate that these mice display increased level of motor learning compared to animals that have not gone through intensive treadmill exercise. This is published in Petzinger et al 2007.

(iii) Intensive treadmill exercise suppresses the intrinsic return of striatal dopamine transporter (DAT) protein. On further analysis tyrosine hydroxylase protein does not appear to be significantly altered by exercise in the MPTP-lesioned mouse. This is published in Fisher et al 2004 and Petzinger et al 2007.

(iv) Intensive treadmill exercise suppresses the expression of dopamine transporter mRNA transcripts in both saline + exercise and MPTP + exercise mice. On further analysis there does not appear to be a significant reduction for the tyrosine hydroxylase mRNA transcript after exercise.

(v) Intensive treadmill exercise causes a normalization of synaptic glutamate to levels seen in non-lesioned mice without exercise. This is published in Fisher et al 2004.

(vi) The administration of AMPA and NMDA receptor antagonists altered the pattern of expression of tyrosine hydroxylase and dopamine transporter mRNA transcription in nigrostriatal dopaminergic neurons as well as the pattern of expression of striatal tyrosine hydroxylase.

(vii) Electrophysiological analysis of dopamine release using fast-cyclic voltammetry indicates increased release of dopamine in the MPTP-lesioned mouse undergoing intensive treadmill exercise compared to MPTP-lesioned or saline. This is published in Petzinger et al 2007.

(vii) Intensive treadmill running increases the D2 receptor mRNA transcript expression but does not alter the expression of D1 receptor subtype. This is published in Fisher et al 2004.

(viii) There is no alteration in the number of SNpc neurons with exercise in either MPTP-lesioned or saline treated mice. This is published in Petzinger et al 2007.

(ix) Immunohistochemical staining with antibody probes against GluR1 and GluR2 and their phosphorylated state shows exercise dependent alterations in the degree and pattern of expression. Results are to be presented at the Society for Neuroscience Annual meeting San Diego, November 2007. A manuscript entitled “Altered AMPA-Receptor Expression with Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury”.

(x) Analysis of mRNA transcripts for the AMPA receptors GluR1 through GluR4 (including their flip and flop isoforms) and the NMDA receptors NR1, NR2A through 2D has been carried out. Results indicate differential expression of some subunits in response to exercise or MPTP-lesioning. Some of these data are presented in this report and are also are to be presented at the Society for Neuroscience Annual meeting San Diego, November 2007. A manuscript entitled “Altered AMPA-Receptor Expression with Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury”.

(xi) Electrophysiological analysis of the relative contribution of AMPA and NMDA receptors in the conductance of currents within the striatum show that there are changes that are exercise dependent. Alterations in LTP and

LTD and the ratio of AMPA to NMDA receptor expression with exercise in the MPTP-lesioned mouse model will be completed in year 4 of this proposal.

(xii) Golgi staining shows some differences in the density of dendritic spines but we are unable to differentiate between the direct population of striatal projection neurons (dopamine receptor D1 containing) and the indirect population (expressing the dopamine receptor D2) using this technique. We have initiated an alternative approach to delineate these populations and analyze the density of dendritic spines with exercise in both saline and MPTP-lesioned mice.

Component Two: Enhancement of neuroplasticity in the MPTP-lesioned nonhuman primate.

(i) The administration of the dopamine agonist Pramipexole induces dyskinesia. This occurs to a lesser degree than that observed with Sinemet.

(ii) Pramipexole and Sinemet increases dopamine levels in both the MPTP-lesioned mouse and MPTP-lesioned squirrel monkey. These changes are most evident in the ventral striatum.

(ii) The addition of microdialysis studies supports the neurochemical HPLC analysis. Specifically treated animals that have undergone repeated microdialysis studies demonstrate an increase in amphetamine induced dopamine release after termination of either Sinemet or Pramipexole treatment.

(iv) Western blot analysis demonstrates a slight increase in TH and DAT protein expression in the dorsal caudate in Pramipexole-treated animals, but no change in DAT expression. Studies examining subregions of the striatum for changes in TH, DAT, DARPP-32, and VMAT protein expression both by western and immunocytochemistry techniques show altered patterns of expression in animals receiving dopamine replacement therapy.

(v) Neurophysiological studies show no difference in dopamine release using fast-scan cyclic voltammetry. Methodological differences between microdialysis and voltammetry may explain why no increase in dopamine was observed in the drug treated groups using voltammetry.

(vi) Neurophysiological studies have demonstrated a low AMPA to NMDA ratio in normal medium spiny neurons of the nonhuman primates. After MPTP lesioning this ratio appears to diverge into two distinct AMPA/NMDA ratio characteristics. In general the population of medium spiny cells appear to diverge either to increase AMPA to NMDA ratio and another medium spiny cell type decreases AMPA to NMDA ration. We found that the input/output relationship was greater for AMPA receptor mediated synaptic currents at 6 weeks after MPTP-lesioning compared to saline control using whole cell voltage clamp. Analysis of animals 9 months after MPTP administration suggests there is normalization of corticostriatal hyperactivity when animals demonstrate full behavioral recovery. Also, that LTD expression at lateral cortico-putamen synapses from the 9-month MPTP-lesioned squirrel monkey is D2 dependent. These neurophysiological studies have been added as an important and informative deviation from the original proposal outline.

(vii) MPTP-lesioned animals demonstrate increased glutamate terminal density relative to saline treated animals. After treatment with either Sinemet or Pramipexole, there is reduced glutamate terminal density occupancy. This finding would support but not confirm that drug treatment facilitates the release of glutamate within corticostriatal terminals. Given the presence of dyskinesia in these treated animals this finding may also support the hypothesis that increased dyskinesia may be in part related to glutamate release.

Key Research Accomplishments for Year Three

One of the key aims of this proposal is to elucidate the mechanisms responsible for improved motor behavior in the MPTP-lesioned mouse model of basal ganglia injury. In our previous report (Fisher et al 2004) studies using immuno-electron microscopy showed that there were alterations in the synaptic occupancy of glutamate such that the MPTP-induced increase is normalized by intensive treadmill exercise. In addition, ongoing electrophysiological studies examining the changes in glutamate receptor expression through pharmacological analysis in *in vitro* slice culture have indicated alterations in the ration of AMPA to NMDA receptor composition of medium spiny neurons as well as a shift in the subunit specific makeup of receptors. To examine the basis of this shift in subunit composition we have carried out an analysis of the pattern of expression of two key AMPA receptor subunits GluR1 and GluR2. The analysis of these subunits is also influenced by our recent findings showing that there is a shift between long-term potentiation and long-term depression with exercise in the MPTP-lesioned mouse undergoing intensive treadmill exercise. The following section describes alterations in the pattern of expression of AMPA receptor subunits GluR1 and GluR2 mRNA transcripts and their flip and flop isoforms using quantitative real-time PCR as well as their protein expression patterns including phosphorylated states using immunohistochemical immuno-staining. These analyses focused on the dorsolateral striatum, a region responsible for motor control.

Results

Treadmill Running Behavior

The time course of improvement in running velocity of both the saline+exercise and MPTP+exercise groups over the 6 weeks (28 days) of treadmill running is shown in Figure 1. Saline+exercise mice in the first week of treadmill running started at a velocity of 14 ± 1.4 m/min that further increased to 22.6 ± 0.3 m/min by the final week. The MPTP+exercise group had a running velocity of 9.2 ± 1.1 m/min during the first week that further increased to 20.5 ± 0.7 m/min in the last week. As shown in our previous papers, there was a significant difference in velocity at week 1 between the saline+exercise and MPTP+exercise groups and this difference was not significant at the completion of the treadmill running regimen [Fisher, 2004; Petzinger 2007).

HPLC Analysis of Striatal Dopamine

HPLC analysis was used to determine levels of striatal dopamine, its metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), and the metabolites turnover ratio, defined as [(DOPAC + HVA)/dopamine]. These data are shown in Table 2. To determine the degree of dopamine depletion by MPTP-lesioning, a subset of non-exercised mice from the saline and MPTP-lesioned groups was analyzed 10 days post-lesioning. At the 10-day time point, the MPTP (48.0 ± 8.4 ng dopamine/mg protein) mice showed significantly lower levels of striatal dopamine compared to the saline group (269.5 ± 24.9 ng dopamine/mg protein) ($p < 0.05$), which represented an 83% depletion. Analysis of dopamine turnover ratio showed a significant elevation in the MPTP group (turnover ratio = 2.3), at the 10-day time point, compared to the saline group (turnover ratio = 0.3) ($p < 0.05$).

HPLC analysis of striatal dopamine at the completion of the 28 days of treadmill running (42 days post-MPTP lesioning) showed that dopamine levels remained significantly depleted in MPTP-lesioned mice compared to saline controls ($F = 229.3$, $p < 0.0001$). There was no significant difference in striatal dopamine levels comparing MPTP+exercise with MPTP sedentary mice. There was a significant effect of exercise on the saline treated group, where saline+exercise mice had a higher level of striatal dopamine compared to saline mice ($F = 7.78$, $p = 0.015$). There were no significant effects of MPTP or exercise, or interaction between these two factors on turnover ratio, with the ratios of MPTP = 0.36, MPTP+exercise = 0.34, saline = 0.26, and saline+exercise group = 0.34.

Analysis of AMPA Receptor Subunits GluR1 and GluR2 Expression

The pattern of expression of mRNA transcripts and proteins for the AMPA receptor subunits GluR1 and GluR2 were determined using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemical staining, respectively. The design of primer sets for qRT-PCR also allowed for the

analysis of the common isoforms of both GluR1 and GluR2 due to alternative transcript splicing, termed flip and flop. In addition, immunohistochemical staining for AMPA-R subunits was determined using antibodies that recognized either the pan or phosphorylated forms of GluR1 and GluR2.

Receptor Subunit GluR1

Immunohistochemical staining of GluR1 subunit expression using a pan-specific antibody shown in Figure 2 showed that the total number of immunoreactive-positive cells decreased in the dorsolateral striatum of the MPTP-lesioned group compared with saline mice, but did not quite reach statistical significance ($F = 4.852$; $p = 0.0587$). There was no significant effect with exercise ($F = 0.04$; $p = 0.846$) and no interaction ($F = 0.063$; $p = 0.809$); specifically, there were no significant differences between saline and saline+exercise groups or the MPTP and MPTP+exercise groups. Analysis of GluR1-immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 1.599$; $p = 0.242$) and between the exercise and non-exercise groups ($F = 1.247$; $p = 0.297$), and there was no interaction ($F = 0.905$; $p = 0.369$).

Immunohistochemical staining for the phosphorylated form of GluR1 was carried out using an antibody that recognizes GuR1~phospho-Ser845 (see Figure 3). There were no significant differences seen in the number of immuno-positive cells between the MPTP-lesioned and saline groups ($F = 0.918$; $p = 0.352$) nor between the exercise and non-exercise groups ($F = 1.493$; $p = 0.239$), and there was no interaction ($F = 0.113$; $p = 0.742$). Analysis of GuR1~phospho-Ser845 immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 1.0$; $p = 0.329$) and between the exercise and non-exercise groups ($F = 0.999$; $p = 0.329$), and there was no interaction ($F = 1.0$; $p = 0.329$).

There was an apparent difference in the intensity of cell body immuno-staining between the different treatment groups within the dorsolateral striatum. Optical density measurements of cell body immunoreactivity captured at high magnification showed no significant difference between MPTP-lesioned mice compared to saline mice ($F = 0.029$; $p = 0.866$), and no significant difference between exercise and non-exercise mice ($F = 1.564$; $p = 0.226$). There was a significant interaction between treatment and exercise ($F = 6.728$; $p = 0.017$), such that exercise led to decreased expression of GluR1~phospho-Ser 845 immunoreactivity within cell bodies of saline treated mice and an increase in expression in cell bodies of MPTP-lesioned mice.

Analysis of mRNA transcript for the pan-GluR1 within the dorsolateral striatum (see Figure 4A) showed that there was a significant decrease in the expression of GluR1 in MPTP-lesioned compared with saline treated mice ($F = 444.0$; $p < 0.0001$) and a significant decrease in the expression of GluR1 in exercise compared to non-exercise mice ($F = 159.0$; $p < 0.0001$). In addition there was a significant interaction between treatment and exercise ($F = 135.9$; $p < 0.0001$), such that exercise led to a decrease expression of GluR1 in the saline group.

Analysis of mRNA transcript for the flip (see Figure 4B) and flop (see Figure 4C) isoforms of GluR1 within the dorsolateral striatum showed an altered pattern of expression. Specifically, there was a significant decrease in the expression of GluR1-flip in the exercise compared to non-exercise groups ($F = 52.05$; $p < 0.0001$). There was no significant effect of MPTP-lesioning on GluR1-flip expression ($F = 0.640$; $p = 0.447$) and no interaction between exercise and MPTP-lesioning ($F = 0.0002$; $p = 0.989$). With GluR1-flop there was a significant decrease in the MPTP-lesioned group compared to saline ($F = 103.3$; $p < 0.0001$). There was no significant effect of exercise on GluR1-flop expression ($F = 3.646$; $p = 0.093$). There was a slight trend for an interaction between exercise and MPTP-lesioning ($F = 3.979$, $p = 0.081$), due to an increased expression in GluR1-flop with exercise in the saline group.

Receptor Subunit GluR2

Immunohistochemical staining of GluR2 subunit expression using a pan-specific antibody shown in Figure 5 showed that the total number of immunoreactive-positive cells increased in the dorsolateral striatum of the MPTP-lesioned group compared with saline mice ($F = 10.370$; $p = 0.012$). There was no significant effect with exercise ($F = 1.133$; $p = 0.318$). There was a trend towards significance in the interaction between exercise and MPTP-lesioning ($F = 4.083$; $p = 0.078$). Specifically, we observed an increase in the expression

of GluR2-immuno-reactivity in the MPTP+exercise mice compared to the MPTP non-exercise mice. Analysis of GluR2-immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 0.358$; $p = 0.566$) and between the exercise and non-exercise groups ($F = 0.178$; $p = 0.684$), and there was no interaction ($F = 0.564$; $p = 0.474$).

In addition to an increase in the number of immunoreactive cells, we also observed morphological changes (degree of arborization) in cells expressing GluR2 (See Figure 5). Specifically, there was a decrease in the arborization of GluR2 immunoreactive cell bodies in MPTP-lesioned compared with saline mice. Interestingly, we observed a dramatic increase in arborization in saline + exercise compared with saline non-exercise mice.

Immunohistochemical staining for the phosphorylated form of GluR2 was carried out using an antibody that recognizes GuR2~phospho-Ser880 (see Figure 6). There was no significant difference in the number of immuno-positive cells between the MPTP-lesioned and saline groups ($F = 2.136$; $p = 0.182$). There was a significant effect with exercise ($F = 19.22$; $p < 0.002$) and there was a significant interaction between exercise and MPTP-lesioning ($F = 5.805$; $p < 0.043$). Specifically, we observed an increase in the expression of immuno-reactivity in the saline+exercise mice compared to the saline non-exercise mice. Analysis of GuR2~phospho-Ser880 immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 2.345$; $p = 0.164$) and between the exercise and non-exercise groups ($F = 0.461$; $p = 0.516$), and there was no interaction ($F = 2.245$; $p = 0.172$). In addition to an increase in the number of immunoreactive cells, we also observed changes in the pattern of immunoreactivity within cell bodies expressing GuR2~phospho-Ser880 between the exercise and non-exercise groups (See Figure 6). The non-exercise group showed more homogeneous staining and the exercise group showed predominant staining in the perimeter of the cell body.

Analysis of mRNA transcript for the pan-GluR2 within the dorsolateral striatum (see Figure 7A) showed that there was a significant decrease in the expression of pan-GluR2 in MPTP-lesioned compared with saline treated mice ($F = 14.83$; $p < 0.005$) and a significant decrease in the expression of pan-GluR2 in exercise compared to non-exercise mice ($F = 9.180$; $p < 0.016$). There was no significant interaction between treatment and exercise ($F = 1.224$; $p = 0.301$).

Analysis of mRNA transcript for the GluR2-flip (see Figure 7B) and GluR2-flop (see Figure 7C) isoforms of GluR2 within the dorsolateral striatum showed an altered pattern of expression. Specifically, there was a significant decrease in the expression of GluR2-flip in the exercise compared to non-exercise groups ($F = 35.90$; $p < 0.0003$). There was also a significant decrease in the expression of GluR2-flip in the MPTP-lesioned group compared to the saline group ($F = 22.47$; $p < 0.0015$). There was no interaction between exercise and MPTP-lesioning ($F = 1.542$; $p = 0.25$). With GluR2-flop there was a significant increase in the MPTP-lesioned group compared to saline ($F = 55.71$; $p < 0.0001$). There was no significant effect of exercise on GluR2-flop expression ($F = 0.04$; $p = 0.843$) and no interaction between exercise and MPTP-lesioning ($F = 2.935$; $p = 0.125$).

DARPP-32

Immunohistochemical staining of DARPP-32 expression was carried out using antibodies that recognize either the pan-specific isoforms (see Figure 8) or the phospho-Thr75 isoforms (see Figure 9). Analysis of DARPP-32 with a pan-specific antibody demonstrated that there was no significant change in the number of immuno-positive cells in MPTP-lesioned group compared to the saline group ($F = 1.334$; $p = .286$). There was a decrease in animals that underwent intensive treadmill exercise compared to non-exercised animals (see Figure 8). This change, however, did not reach statistical significance ($F = 2.47$; $p = 0.16$). There was no significant interaction between exercise and MPTP-lesioning ($F = 0.002$; $p = 0.97$). Further analysis using the antibody specific for the phospho-Thr75 isoform of DARPP-32 demonstrated no significant change in the number of immuno-positive cells in the MPTP-lesioned group compared to saline animals ($F = 0.315$; $p = 0.590$), no significant change in the exercise compared to the non-exercise group ($F = 0.167$; $p = 0.693$), and no significant interaction ($F = 1.599$; $p = 0.242$).

Key Research Accomplishments for Year Four

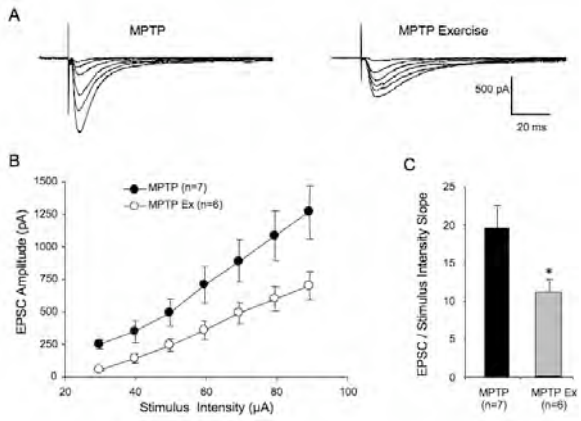
Overview:

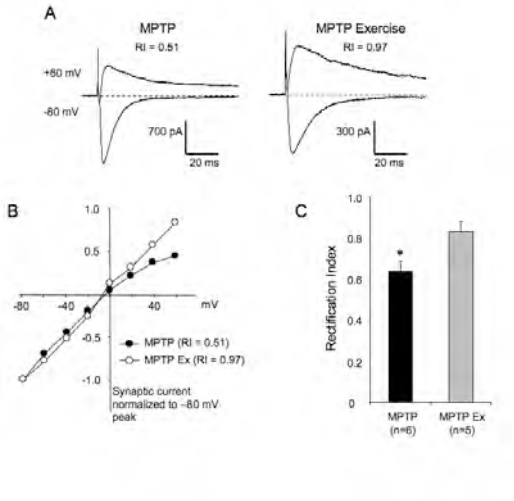
The overall goal of this NETRP grant is to understand to role of experience (exercise) and dopamine replacement therapy on neuroplasticity of the injured basal ganglia utilizing the MPTP-lesioned mouse and MPTP-lesioned nonhuman primate models of PD. Examining the effects of exercise on the MPTP-lesioned mouse, we have focused on investigating the role of AMPA-R subtype of glutamate receptors in experience dependent plasticity of the injured basal ganglia. The AMPA-R is responsible for fast excitatory neurotransmission in the CNS and is critically involved in leaning and encoding such as occurs with long-term depression (LTD) and long-term potentiation (LTP) New electrophysiological data support alterations in the AMPA-R, and specifically an increase in GluR2 expression with exercise. In addition, we are investigating whether changes in AMPA-R are specific to certain cells of the striatum. Using a transgenic mouse cell line that aids us to identify medium spiny neurons within the striatum that are primarily projecting to the direct pathway (D1 dopamine receptor expressing) or to the indirect pathway (D2 dopamine receptor expressing), we are beginning molecular and neurophysiological studies to explore whether changes in AMPA are specific to either pathway. We are also beginning to explore downstream effector molecules that may be modulated by both dopamine and glutamate through either of their respective receptors. In addition, we have also begun to validate our findings of an exercise -induced increase of the dopamine D2 receptor by utilizing PET imaging and a novel D2 receptor ligand, called F18-Fallypride. We have begun to translate our exercise findings in the MPTP-lesioned mouse to a study examining the effects of high intensity treadmill exercise in individuals with PD. This has already led to the publication of one manuscript showing that intense exercise can improve motor function in individuals with PD and lead to changes in cortical excitability. We are currently undergoing a PET study using F18-Fallypride to examine whether high intensity treadmill exercise leads to an increase in dopamine D2 receptor expression in individuals with PD. Exercise induced increase in dopamine D2 receptor may allow for compensation in the presence of low levels of dopamine, as occurs in PD. Given the link between neuroplasticity, the glutamatergic system, and morphology, we have also initiated a study to examine exercise-induced alterations in dendritic spine density. Additional studies carried out this year was a study comparing voluntary to forced exercise in the MPTP-lesioned mouse to begin to examine the question of stress and exercise, and finally a study beginning to examine mood and cognitive features with neurochemical correlates in the MPTP-lesioned mouse. The later study will allow us to begin to address the role of exercise in mood and cognition in the MPTP mouse model of basal ganglia injury.

In the MPTP-lesioned nonhuman primate our focus has been to understand the role of dopamine replacement therapy on the neuroplasticity of the striatum, and to examine for regional differences in both dorsal and ventral striatum. Our data suggests that dopamine replacement therapy leads to a modest increase in dopamine levels (HPLC and microdialysis) and proteins important for dopamine biosynthesis and storage. These subtle changes appear to be most evident in the ventral striatum where dopamine terminals are characteristically less affected by the MPTP-induced injury. Behaviorally these animals, as predicted, develop pronounced dyskinesia with either dopamine agonists or L-dopa, and so this modest benefit in restoring dopamine within the ventral striatum must be weighed against this disabling drug-induced motor complication. While the majority of the western blotting has been completed to examine these proteins of interest, our justification for the no-cost extension is to allow us to complete their analysis and prepare a manuscript for publication. Given our findings that the AMPA-R subtype of glutamate appears to be important in experience dependent plasticity of the injured basal ganglia, we are examining alterations in the AMPA-R after dopamine replacement in the MPTP-lesioned nonhuman primate. Our previous EM studies support changes in glutamatergic neurotransmission, as measured by glutamate terminal immunolabeling, in the MPTP-lesioned NHP treated with dopamine replacement therapy. Most of the westerns examining changes in AMPA-R subunits are completed but the additional no-cost extension will allow us to complete the analysis of the study. Our previous year three and during year four neurophysiological studies in the MPTP-lesioned nonhuman primate have supported changes in the AMPA-R and GABA during the intrinsic recovery process of the striatum. This neurophysiological study has provided new insight regarding long-term alterations in glutamatergic neurotransmission during the intrinsic recovery period of the injured basal ganglia.

Details: MPTP-lesioned mouse

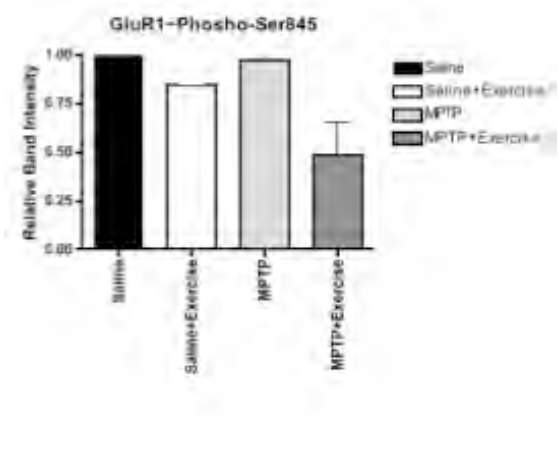
(1) **Additional supportive evidence for the AMPA receptor (especially the GluR2 subunit) in experience-dependent plasticity specifically the role of intensive treadmill exercise in the injured basal ganglia**: In year three we reported that high intensity treadmill exercise in the MPTP-lesioned mouse led to an increase in GluR2, and GluR2 phosphoserine882 expression in the MSNs of the striatum using immunocytochemistry. No exercise -induced detectable changes were noted in the GluR1 subunit. In year 4 we have conducted neurophysiological studies within the MSNs of the striatum to support our molecular findings. Specifically, neurophysiological studies using whole cell voltage clamp demonstrated that treadmill exercise leads to both a reduction of the size of the Excitatory Post-Synaptic Currents (EPSCs) in the medium spiny neurons (input-output curves) and a loss of polyamine sensitive inward rectification, both supportive of a GluR2 increase. Within the final phase of this grant we are also preparing to examine the exercise-induced alterations in the relative contribution of AMPA and NMDA in the MSNs of the striatum. ($p < 0.004$) In the top Panel, A: Example of EPSCs evoked from dorsolateral corticostriatal synapses in brain slices take from MPTP alone and MPTP + exercise mice. EPSCs were produced by the stimulation intensities shown in the x-axis of B. B: plot of input (stimulation intensity)- output (EPSC amplitude) for corticostriatal synapses from MPTP treated mice. C: Bar graph of slope calculated for the input-output relationship for corticostriatal synapses as shown in B. In the lower panel, A: Example of EPSCs evoked in dorsolateral striatal neurons at holding potentials of -80 and +60 mV from MPTP alone and MPTP + exercise mice (stimulus intensity =80uA). B: Current-voltage plots of synaptic currents evoked for the cells illustrated in A. Synaptic currents were normalized to the peak synaptic current evoked at -80mV for ease of presentation. C: Measurement of rectification in current-voltage relationship, or the rectification index (RI) (ratio of synaptic conductance at +60mV vs -80 mV). Synapses from MPTP mice demonstrated a significantly lower RI (more rectification) versus MPTP + Exercise mice. ($p < 0.03$)





(2) Development of technique for the preparation of enrichment for synaptic proteins called synaptoneurosomes:

Analysis of the pattern of protein expression of the AMPA receptor subunits GluR1, GluR2, and their phosphorylated states was carried out using immunohistochemistry approaches in fixed tissue sections. Our analysis was able to report relative differences between treatment groups (i) saline, (ii) saline + exercise, (iii)

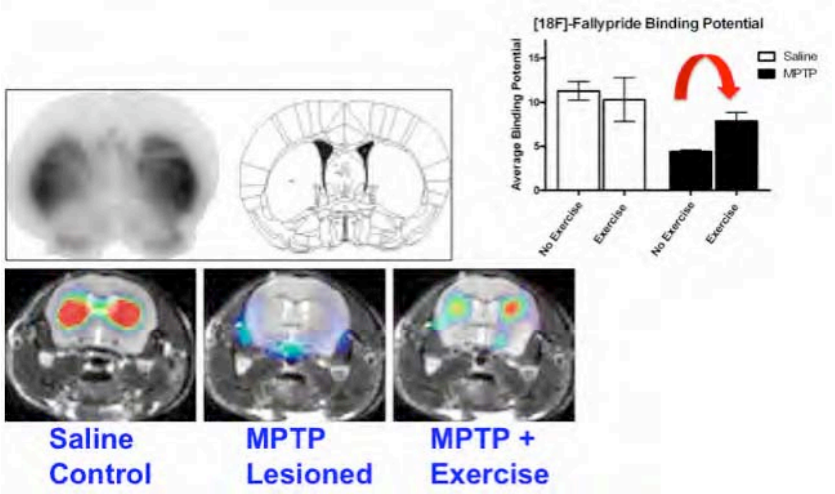


MPTP, and (iv) MPTP + exercise. Many of these findings were reported in the Year 3 Annual Report as well as the manuscript from our lab that is included as a pdf in the Appendix of this Year 4 Annual Report.

While we examined protein changes in cell bodies expression, the primary role of AMPA receptors in regulating the glutamatergic properties of a cell is at the level of the synapse. Therefore our next series of experiments will entail examining the expression of these proteins within synaptic contacts of the striatum. The technique we employed involves the enrichment of synapses, termed synaptoneurosomes, in which homogenized tissue of interest is passed through a series of micro-grids thus removing cell bodies and their cytoplasm. A Figure outlining this methodology is presented. Proteins prepared for synaptoneurosomes were subjected to western immunoblots to determine the relative degree of expression of proteins of interest. At the time of the writing of this report we are engaged in examining the relative degree of expression of the AMPA receptor subunits GluR1, GluR2 and their phosphorylated states to determine if changes observed using ICC are also seen with synaptoneurosome preparations. Differences between ICC and western immunoblotting may reflect site of occupancy of these proteins (receptors) to either the synapses or cytoplasm.

Synaptoneurosome Preparations from Striatal Tissue Method: Synaptoneurosomes are prepared from by the method of Johnson et al. 1997 and also used by others (Banko et al. 2004,Villasana et al. 2006) with slight modifications. This procedure is rapid and gentle requiring about 40 minutes. Brain tissue is homogenized with a Teflon-homogenizer (4 strokes at 1000 rpm) in buffer (1/10wt/vol), containing 0.35M sucrose pH7.4, 10mM HEPES, 1mM EDTA, 0.25mM dithiothreitol, 30U/ml RNAse inhibitor and a protease inhibitor cocktail (Roche, Inc). Cell debris and nuclei are removed by centrifugation at 1000g for 10 min at 4°C yielding pellet P1 and supernatant S1. The S1 fraction is passed through a series of 4 nylon mesh screens with decreasing pore size finishing with passage through a 5-micron screen. The final filtrate is resuspended in 3 volumes of buffer without sucrose for mRNA extraction, or appropriate buffer for subsequent assays, and centrifuged at 2000g, for 15 minutes at 4°C. For detection of nuclear contamination, smears of synaptoneurosome pellets are air-dried onto microscope slides then fixed in ice-cold acetone for 5 minutes. The pellet is assayed for protein content and either snap-frozen or suspended in incubation buffer. These preps are then utilized in the western immunoblot and co-IP techniques to detect proteins of interest.

(3) Analysis of the pattern of expression of the dopamine D2 receptor using in vivo PET-imaging:

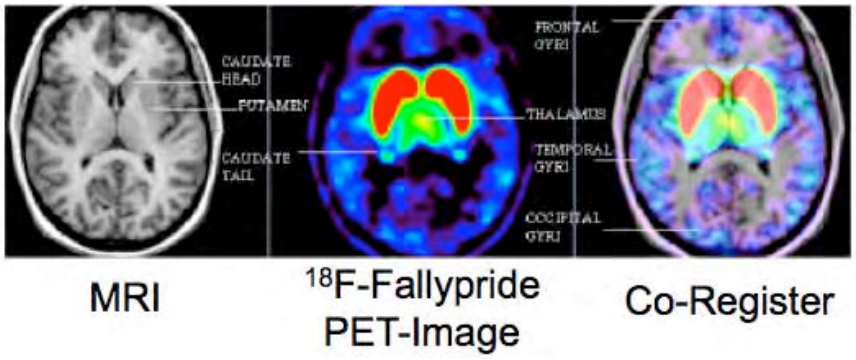


Using a novel D2 receptor ligand, called F18-Fallypride we have been able to examine exercise-induced alterations in the dopamine D2 receptor. Preliminary studies in PET suggest that exercise leads to an increase in the D2 receptor, and that these exercise effects appear to be specific to the MPTP-lesioned mouse as these changes are not observed in exercise + saline animals. The attached Figure shows representative images of receptor autoradiography and anatomical representation of 18F-fallypride binding (upper left panel), co-registered images of MRI and 18F-fallypride PET-imaging of representative mice from saline, MPTP alone, and MPTP+exercise upon completion of 28 days of treadmill exercise (lower left 3 panels), and the

graph shows accumulated data from all groups (n=6 per group) demonstrating increased 18F-fallypride binding potential in the MPTP+exercise groups (upper right panel). These data demonstrate and are consistent with elevated expression of the DA-D2R with intensive treadmill exercise in the MPTP-lesioned mouse model of Parkinson's disease. These data are now include in a manuscript in the final stages of completion that will be submitted by August 2009.

(4) Proof of principle for analysis of dopamine D2 receptor binding potential in patients with Parkinson's disease:

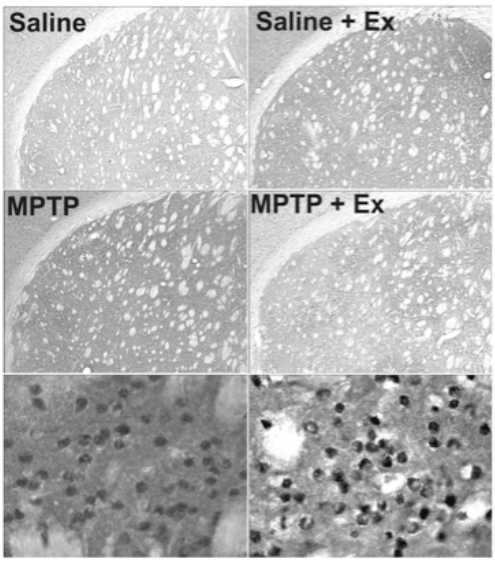
We are beginning to translate findings from the MPTP + exercise study to a human study examining exercise induced alterations in the dopamine D2 receptor in individuals with PD. The impact of this translational study is enormous because it allows us to test the hypothesis that exercise leads to compensatory changes in the dopamine signal in patients with PD, and thus may facilitate a form of disease modification.



See Year 5 section for update of this study.

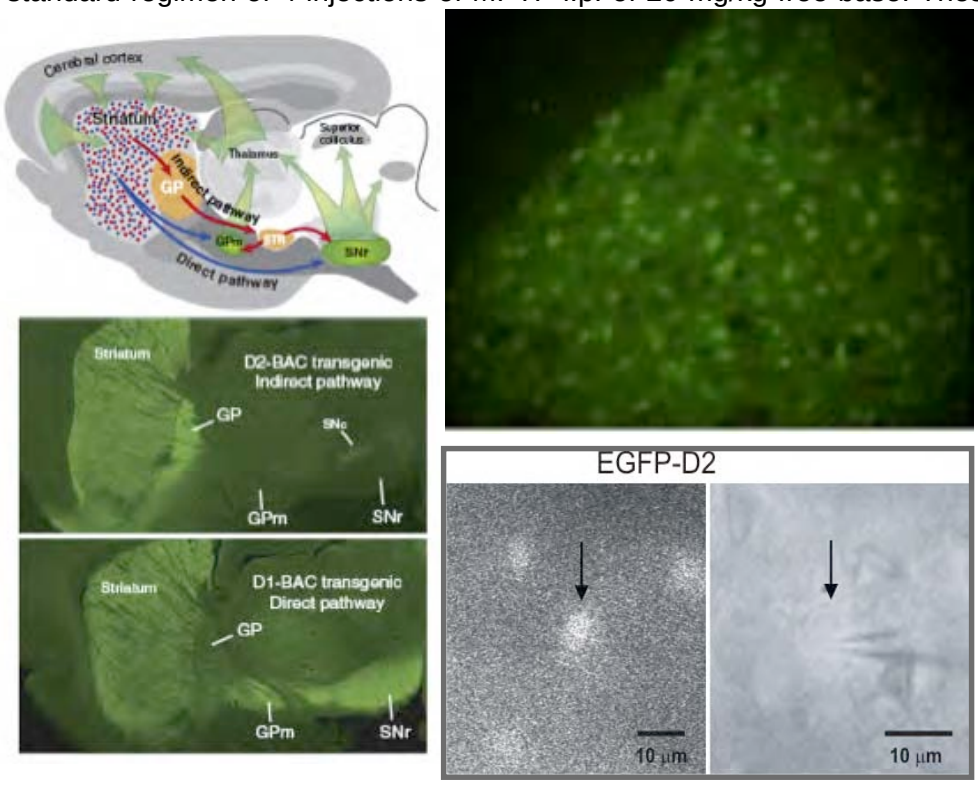
(5) Analysis of the transcription factor CREB:

Within medium spiny neurons the transcription factor CREB and its activated state phospho-CREB can act as mediators of glutamatergic and dopaminergic neurotransmission. This factor is able to regulated a wide spectrum of target genes and may be one means by which experience-dependent plasticity influence the recovery of motor behavior seen in our model. To examine the pattern of expression of CREB and phospho-CREB in our model of basal ganglia injury and exercise we examined the expression of these proteins in the dorsolateral striatum of mice from all groups including (i) saline, (ii) saline + exercise, (iii) MPTP-lesioned, and (iv) MPTP + exercise. After 30 days of intensive treadmill exercise, mice were perfusion fixed and brain tissue sections through the mid-striatum prepared for immunohistochemical analysis. Our analysis of the number of medium spiny neurons and the intensity of protein expression indicate elevated expression of phospho-CREB in the striatum of MPTP + exercise mice. Studies will be completed in the last phase of this study using western immunoblot analysis to determine the relative pattern of expression between the four groups of mice. This Figure is a representative analysis of expression of the phosphorylated form of CREB highlighting the dorsal striatum. The bottom panels are a higher magnification of images from MPTP-lesioned (left bottom panel) and MPTP + exercise (right bottom panel) showing increased expression in large neurons within the dorsolateral striatum.



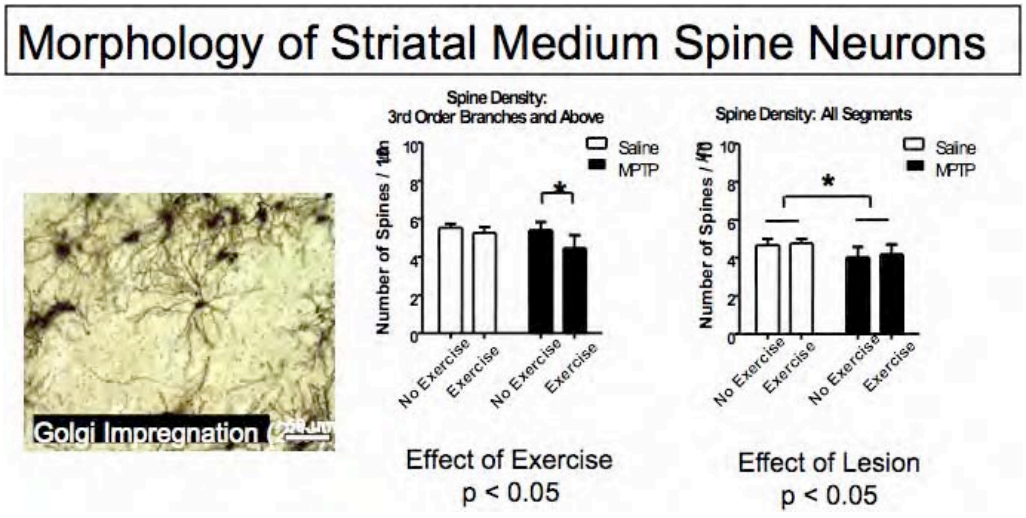
(6) Acquisition of the BAC-D2-eGFP protein:

The changes in the dopamine D2 receptor leads us to investigate whether the changes in this receptor are localized to either the direct (predominantly D1 receptor containing) or indirect (predominantly D2 receptor containing) pathways of the striatum. To address this issue we have obtained a transgenic mouse strain in which the enhanced green fluorescent protein (eGFP) is under the control of the D2 promoter. This transgenic strain, termed BAC-D2-eGFP, allows us to directly perform electrophysiological studies as we have conducted as outlined in this report. This strain was obtained from the laboratory of Michael Levine (UCLA) during the 4th year of this grant. At the writing of this report we have begun to breed these transgenic mice in order to have sufficient numbers for studies, have characterized PCR-based screening methods for genotyping mice in our colony, have verified the these mice express eGFP and therefore display green florescence in striatal neurons that can then be subjected to electrophysiological studies, and we have administered MPTP using our standard regimen of 4 injections of MPTP i.p. of 20 mg/kg free-base. These mice are susceptible to MPTP as assessed through measurement of striatal dopamine, TH-immunoreactivity, and cell loss in the substantia nigra pars compact based on unbiased stereological counting techniques. The aim of studies is to now use this strain molecular, morphological, and electrophysiological studies to localize the changes we observe in both dopamine D2 receptor and the GluR2 AMPA receptor to the indirect pathway. It is possible that changes in these receptors may occur on other anatomical sites within the striatum including the direct pathway, pre-synaptic sites (nigrostriatal or corticostriatal), or interneurons. The Figure illustrates some of these points the left panels highlight the striatal pathways and below the cartoon the BAC-D2-eGFP (indirect pathway) and BAC-D1-eGFP (direct pathways) are illustrated (images from Gerfen 2008). The right top panel shows GFP cells in a striatum from a mouse in our colony and the bottom panel is an image depicting a striatal neuron and the juxtaposition of electrode for recordings.



(7) Analysis of dendritic spine density:

Our findings with elevated expression of both the dopamine D2 receptor and GluR2 AMPA receptor suggests that such changes may be accompanied by changes in the morphology of dendritic spines in medium spiny neurons. To investigate this potential relationship between intensive treadmill exercise and recovery of motor behavior we are examining the density and morphology of dendritic spines on striatal neurons. Brain tissues from mice in groups from saline and



MPTP with and without exercise were prepared for Golgi impregnation. In conjunction with computer assisted image analysis using the program Bioquant and an Olympus BX-51 microscope with motorized stage we are now carrying out an extensive analysis of medium spiny neuron morphology. While we are able to detect some differences between MPTP-lesioning and exercise we anticipate completion of this phase during the no-cost extension period of this grant by examining more mice from all groups and the analysis of the type of spine based on its morphology. As an alternative approach we are planning to investigate whether the BAC-D2-eGFP mice, where expression of GFP in striatal neurons of the indirect pathway may be utilized with confocal microscopy to analyze spine density. This possibility will be explored in the last phase of this grant.

(8) Examination of affective behavior in the MPTP-lesioned mouse model:

A key question in the studies in this grant has been the effects of intensive treadmill exercise on the recovery of motor behavior. While our exercise regimen leads to enhanced recovery of motor features we though an important corollary of these studies was to determine if MPTP-lesioning and exercise have effects on non-motor features including affective behaviors (anxiety and depression), associative memory, and fear conditioning. This is important in light of the fact that patients suffering from PD also display affective behavioral changes, which in themselves can be very debilitating, and that these features are thought to be due to dysfunction in both dopamine and serotonin neurotransmission. Therefore, a major question is whether the MPTP-lesioned mouse model of basal ganglia injury also displays non-motor behavioral features that can be detected. For these studies, mice were administered saline or MPTP (4 injections of 20 mg/kg free-base, 2 hour intervals) and subjected to a battery of behavioral tests at both 7 and 30 days after MPTP-lesioning that included associative memory (social transmission of food preference), fear-conditioning, anxiety (light-dark preference, and hole board), and depression (tail suspension and sucrose preference). Our overall conclusion is that the MPTP-lesioning regimen that we use in 8 to 10 week old C57BL/6 mice manifests deficits in impairment of associative memory, increased extinction of fear-conditioning, but no detectable increase in anxiety and depression. While this model serves as an excellent means to study dopamine depletion and basal ganglia function the precise parameters underlying some of the non-motor features as seen in PD have yet to be established.

This manuscript is published in the journal Neurobiology of Disease and a copy of this manuscript in pdf form is included in the Appendix of this report.

(9) Comparison of forced (intensive treadmill) exercise with voluntary home-cage wheel running:

Studies in this grant have focused on intensive treadmill exercise as a means to enhance the recovery of motor behavior features. While we have not examined the precise role of stress in our exercise paradigm that employs running on a motorized treadmill the question arises as to whether voluntary running on a running-wheel in the home cage is different from the results we observe. Initial results indicate based on thymus weighs as an indicator of stress that there is no difference in stress levels between forced treadmill exercise and voluntary running-wheel at the completion of a 30 day exercise program. CORT levels are now being analyzed to verify this finding.

Year Five:

These studies are now in the stages of assembly into a manuscript to be submitted for publication. We have analyzed dopamine, serotonin, and their metabolites in brain regions including the striatum, basal lateral amygdala, frontal cortex, ventral mesencephalon, as well as corticosterone levels in mice subject to either forced treadmill exercise, voluntary running in their home cage, or no exercise. Our overall results show modest changes in serotonin levels between the treatment groups corresponding to differences in corticosterone levels. The manuscript describing these findings is now in preparation and should be ready for submission by September 2009.

The following section outlines experimental details of studies carried out in the MPTP-lesioned nonhuman Primate. Overall there are no additional changes between Years Four and Five reports. The only additional data that has been generated in Year Five has been western immunoblot analysis of striatal proteins for AMPA receptor subunits GluR1 and GluR2 from tissues obtained from these animals. It is our goal to assemble the following electrophysiological data we have obtained with protein expression analysis to submit a manuscript for publication. Due to restraints on time in completion of studies from Component 1 mouse studies in this grant we have not yet achieved assembly of manuscripts for Component Two, nonhuman primate studies. We anticipate submission of a manuscript describing our electrophysiological findings over the next several months.

Overall Conclusions

1. Vesicular dopamine release, measured via cyclic voltammetry, demonstrates that striatal dopamine signal is significantly decreased in the MPTP treated squirrel monkey compared to saline control even at one year. Animals display full behavioral recovery at one year despite the low dopamine levels within the putamen. (Figure 2)

2. Parkinsonian-like squirrel monkeys display excessive corticostriatal excitation at 8 weeks post MPTP treatment as demonstrated by input/output curves (fig. 3). However, cortical drive (as determined by input/output data) and behavior returns to normal by one year post MPTP.

3. Medial to lateral differences in short and long-term plasticity exists at corticoputamen synapses of the squirrel monkey. One year post-MPTP lateral synapses show enhanced LTD in spite of the lack of return of dopamine. This property may reflect an increase in the sensitivity and expression of D2 receptors laterally. (Figures 4, and 5)

4.Increased $GABA_A$ receptor mediated inhibition is seen in squirrel monkeys treated with MPTP one year earlier as compared to animals treated with MPTP 8 weeks earlier (figure 6)

5. No differences were seen between control, saline injected, 8 week post MPTP and one year post MPTP synapses in the $EPSP_{NMDA}/EPSC_{AMPA}$ ratio (Figure 7). There was a trend toward greater NR2B subunit expression in animals treated with MPTP 8 weeks earlier.

6. MPTP treated animals showed greater sensitivity to GYKI than saline treated animals. This increased sensitivity may be due to either an increase in the absolute number of AMPA receptors and/or alterations in AMPA-R subunit composition (figure 8).

As a service for review of this Final Report we have included the data figures pertaining to findings in the nonhuman primate in the following section. It is our intention in the upcoming month to assemble these data in a manuscript for publication either as its own separate report or in addition to electrophysiological studies yet unpublished in the mouse model of MPTP-lesioning.

(1) Electrophysiological Studies in the nonhuman Primate

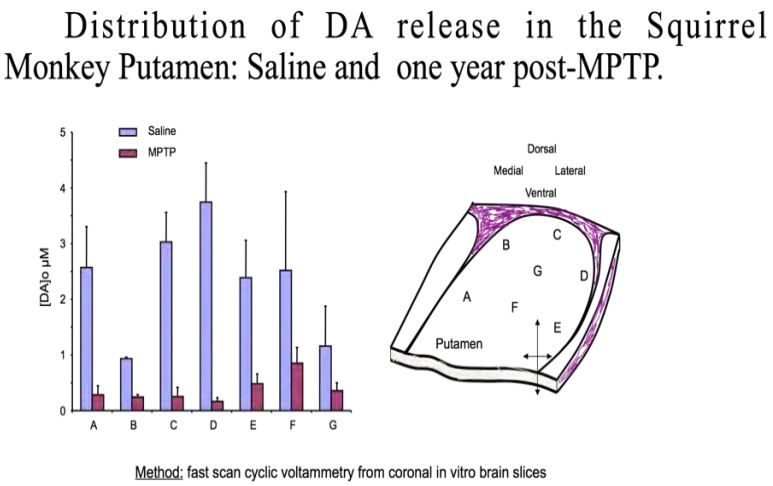


Fig. 2. Lack of recovery in putamen dopamine release one year after MPTP lesions in the squirrel monkey as revealed by cyclic voltammetry. Coronal slices made from the anterior putamen were sampled with electrodes placed at seven sites (A-E), as illustrated. Single slices were examined from each animal (2 control and 4 MPTP). The MPTP animals showed full behavioral recovery at the one-year post-MPTP sampling time in spite of the lack of return in evoked dopamine release. Regional differences were seen in evoked dopamine release as reported earlier for the primate by Cragg (2003).

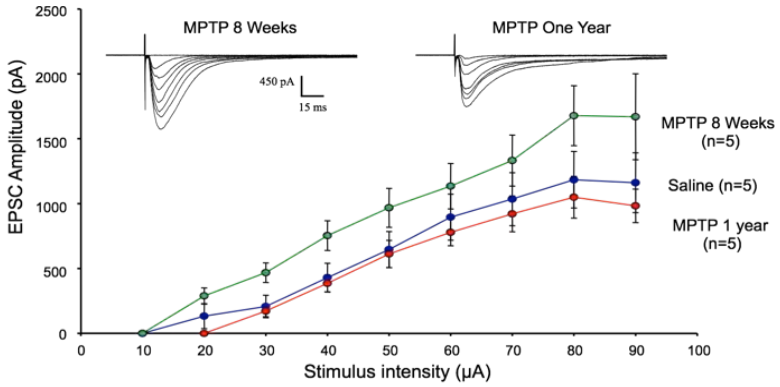


Fig. 3. Behavioral recovery is associated with a reduction in corticostriatal synaptic strength one year after the MPTP lesion. Input output analysis suggests early and long-term post-MPTP recovery results in a change in the strength of corticostriatal synapses. Larger excitatory postsynaptic currents (EPSCs) were evoked in animals injected with MPTP 8 weeks earlier as compared to saline injected animals when identical stimulation intensities were delivered to the corpus callosum (input/output relationship) (n=5 for each group, repeated measures ANOVA, p=0.18). Interestingly, the input-output relationship for animals injected with MPTP a year earlier returned to the level seen in control, saline injected squirrel monkeys. A repeated measures ANOVA performed for the input-output relationship of corticoputamen synapses between the 8 week and one year post-MPTP revealed a recovery trend across all stimulation intensities (p=0.06, n=5 for both MPTP groups).

Synaptic Plasticity at Cortico-putamen Synapses from the Squirrel Monkey: Saline and 1 year after MPTP.

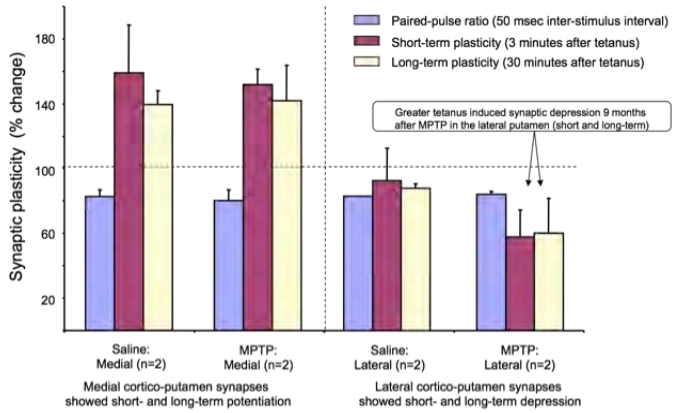


Fig. 4. Corticoputamen synapses from the squirrel monkey show region-dependent forms of synaptic plasticity, with increased LTD expression occurring in the lateral putamen of animals injected with MPTP one year earlier. Medial corticoputamen synapses showed short- and long-term synaptic potentiation (STP & LTP) and lateral corticoputamen synapses showed short- and long-term synaptic depression (STD & LTD) in both control animals and in animals injected with MPTP one year earlier (n=2 for each bar in the graph). While not significant, the tendency toward greater LTD in the MPTP-recovered group is particularly interesting in light of the lack of dopamine seen in the putamen of these animals (see fig. 2).

Examples of tetanus induced changes in the amplitude of cortico-putamen excitatory synaptic potentials (EPSP) in the squirrel monkey: One year post MPTP vs Saline.

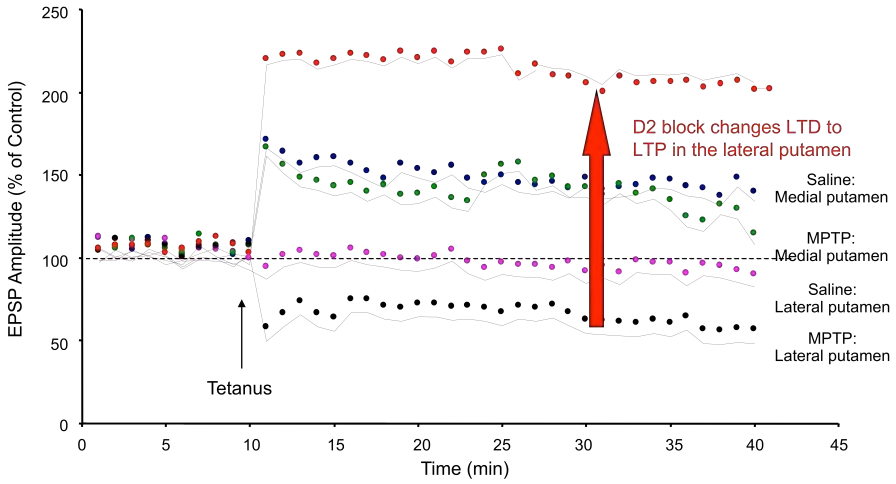


Fig. 5. Time course of tetanus-induced plasticity at corticoputamen synapses obtained from individual neurons from the squirrel monkey. The data illustrates the medial trend of LTP and the lateral trend of LTD. Interestingly, block of D2 receptors with sulpiride enables the expression of LTP in the lateral putamen in spite of the marked decline in putamen dopamine observed one year after MPTP (see Fig. 2). A possible explanation for this effect of D2 receptor block in the MPTP treated animals is a compensatory increase in the activity of putamen D2 receptors when dopamine is depleted

Behavioral recovery is associated with MPTP induced changes in $GABA_A$ receptor-mediated inhibition in putamen neurons from the squirrel monkey

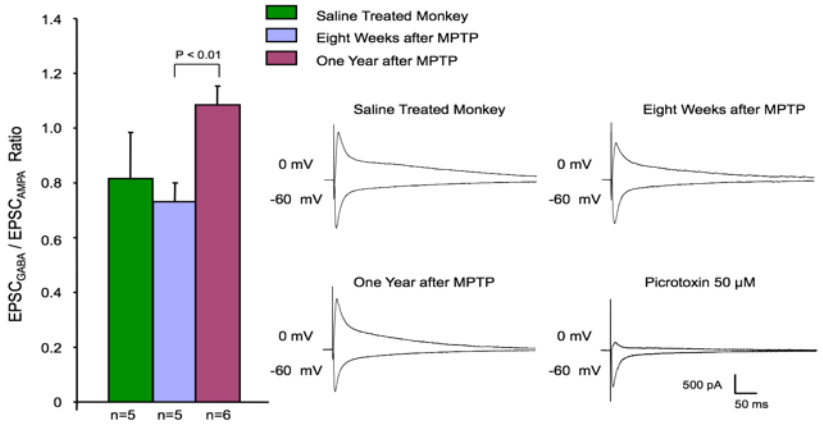


Fig. 6. Whole cell voltage clamp of squirrel monkey putamen neurons revealed a post-MPTP recovery difference in the $GABA_A$ -mediated inhibitory postsynaptic current (IPSC) evoked by stimulation of the corpus callosum. Putamen neurons were voltage clamped at –60 mV to measure AMPA receptor-mediated EPSCs **(-60 mV was chosen because it approximates the reversal potential of $GABA_A$ receptor mediated IPSCs)** and at 0 mV to measure $GABA_A$ receptor mediated IPSPs **(0 mV was chosen since this is the reversal potential for glutamate receptor mediated synaptic events).** Results obtained using this method of isolating EPSCs and IPSCs are illustrated in the traces to the right. Addition of the $GABA_A$ receptor antagonist picrotoxin blocked the IPSC evoked in the neuron from the animal treated with MPTP one year earlier.

The bar graph to the left plots the ratio of the amplitude of the ratio of the IPSC measured at 0 mV to the amplitude of the EPSC measured at –60 mV. No difference in the IPSC/EPSC ratio was seen between control, saline injected monkeys and those treated with MPTP 8 weeks earlier. However, a difference was observed in the IPSC/EPSC ration between monkeys injected with MTPT 8 weeks earlier and those allowed to recovery for one year ($p<0.01$).

Recovery from MPTP does not change the relative contribution of NMDA versus AMPA receptors to cortico-putamen evoked synaptic currents in the squirrel monkey

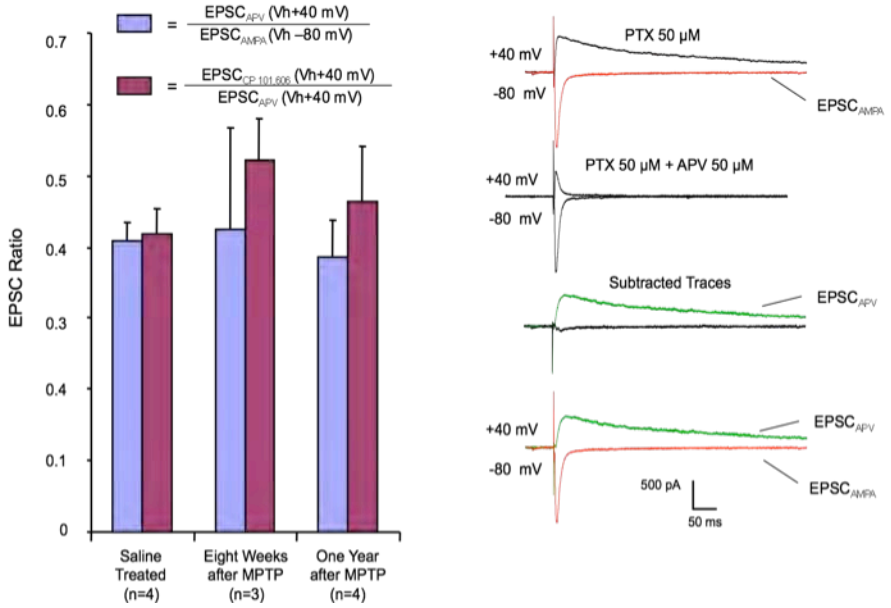


Figure 7. Whole-cell voltage clamp methods were used to isolate NMDA and AMPA receptor mediated synaptic currents evoked by cortico-putamen stimulation in the squirrel monkey. Methods and outcomes are illustrated in the traces to the right. Picrotoxin was added to block $GABA_A$ receptor mediated inhibition. Cells were then voltage clamped at –80 mV to isolate the AMPA receptor mediated EPSC and at +40 to maximize the expression of the NMDA receptor mediated EPSC. APV was added to block the NMDA receptor EPSC and the post-APV traces were obtained by computer subtraction at a holding potential of +40 mV. This value was then plotted versus the AMPA receptor mediated EPSC recorded at –80 mV ($EPSP_{NMDA}/EPSC_{AMPA}$ – blue bars in graph). No differences were seen between control, saline injected, 8 week post MPTP and one year post MPTP synapses in the $EPSP_{NMDA}/EPSC_{AMPA}$ ratio. Slices were exposed to the selective NR2B antagonist CP101,606 prior to APV to determine if the relative contribution of the NR2B subunit changed with recovery from MPTP lesioning in the squirrel monkey. No differences were observed, but a trend toward greater NR2B participation was seen in the monkey injected 8 weeks earlier with MPTP (purple bars in graph).

Recovery from MPTP increases the sensitivity of striatal glutamate receptor-mediated EPSCs to GYKI 52466 .

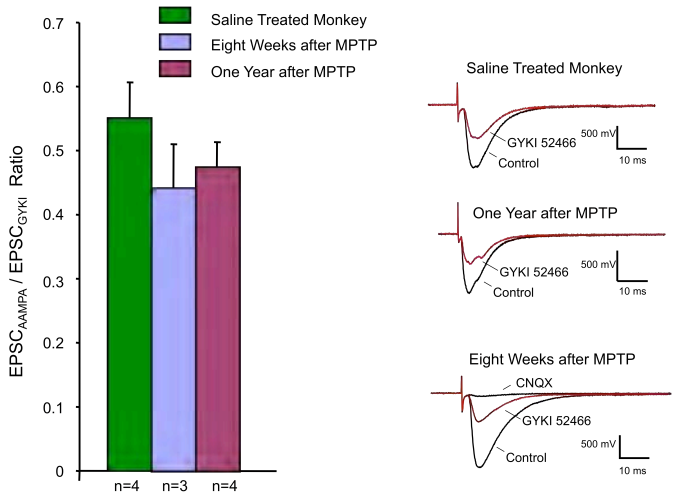


Fig. 8. Whole cell voltage clamp methods revealed corticostriatal AMPA receptor-mediated EPSCs from MPTP treated monkeys showed greater sensitivity to the selective GluR1-6 antagonist GYKI 52466. While the small sample size does not lend itself to testing differences, the trend was for greater block of the AMPA receptor mediated EPSCs by GYKI 52466 in MPTP treated monkeys (8 week and one year post injection).

Key Research Accomplishments for Year Five

The following section describes additional studies within the original Specific Aims of this grant that have been completed.

As described in Year 4 we have now established the technique of preparation of synaptoneurosomes, which is an enrichment of synaptic structures. We have used this preparation in conjunction with western immunoblotting to validate studies using whole tissues homogenates from striatal tissues. Results from this technique are included in a manuscript now In Press (VanLeeuwen et al, 2009; see appendix). This paper reports alterations in the pattern of expression of the AMPA-R subunit GluR2 and its phosphorylated states in MPTP-lesioned mice subjected to intensive treadmill exercise. as well included in a manuscript in the final stages of preparation examining the pattern of expression of DA-D2R in MPTP-lesioned animals subjected to intensive treadmill exercise (Vuckovic et al, 2009).

Specific Aim 1: ***Study 4:*** *The pattern of expression of subunits for both the NMDA and AMPA receptor subtypes and their phosphorylated state will be determined using western immunoblotting, immunocytochemistry and in situ hybridization histochemistry*.

Immuno-electron studies in Study 3 of Specific Aim 1 indicated increased in levels of glutamate immunolabeling within corticostriatal terminals, that were normalized after intense treadmill exercise to that of saline treated animals (Fisher et al 2004, see appendix).

Immunocytochemical analysis of striatal tissue demonstrated that exercise led to a significant increase in the number of GluR2 subunits (AMPA-R) positive neurons, and its phosphorylated form (serine 880) in MPTP treated animals (VanLeeuwen et. al 2009, see appendix). We observed no changes in the pattern of expression of GluR1, 3 or 4 subunits. Western blot analysis of synaptoneurosomal preparations of the dorsal striatum supported findings observed with ICC studies. Specifically we observed a significant increase in GluR2 subunit after exercise in MPTP treated mice, but no change in GluR1 subunit. In situ hybridization, also demonstrated an exercise induced increase in GluR2 mRNA transcript. These molecular findings in GluR2 subunit expression were further analyzed using a neurophysiological approach to examine for the synaptic and functional expression of this subunit within the medium spiny neurons. Specifically GluR1 containing AMPA-Rs (lacking subunit GluR2) demonstrate polyamine sensitivity and inward rectification, and GluR2 containing AMPA-Rs are insensitive to polyamine and demonstrate a linear current-voltage relationship and no rectification. In support of our molecular studies, after MPTP administration we observed a predominance of inward rectification in our recordings of MSNs within the dorsal striatum, and after exercise there was loss of rectification (supportive of GluR2 containing AMPA-Rs). Thus our neurophysiological studies supported our molecular findings. The conclusion of these studies was that exercise led to changes in GluR2 expression and in the synaptic expression of GluR2 containing AMPA-Rs, supporting the role of AMPA-Rs in mediating experience-dependent neuroplasticity in the injured basal ganglia (VanLeeuwen et al 2009).

Additional neurophysiological studies were carried out to examine the effects of exercise on the spontaneous excitatory post-synaptic currents (sEPSCs) of the MSNs. We observed an increase in the amplitude of sEPSCs after MPTP administration, a finding previously reported in the literature. Interesting after intensive treadmill exercise we observed a reduction in the amplitude of the sEPSC to that of saline treated animals. Changes in sEPSC amplitude could be reversed with an AMPA blocker supporting that sEPSC was AMPA mediated. We observed no changes in presynaptic glutamate release, using paired-pulse studies, to explain this difference in amplitude. We hypothesized that exercise induced changes in sEPSC amplitude may be explained in part by an exercise-induced increased in the synaptic expression of GluR2 containing AMPA-R. GluR2 containing AMPA-R flux less calcium and elicit a smaller response (decreased synaptic strength) to glutamate binding then GluR2 lacking AMPA-Rs. The latter hypothesis was supported by a neurophysiological study that demonstrated a reduction in the evoked input-output curve in exercise animals compared to non-exercise animals (VanLeeuwen et al, 2009). Given the fact that the dopamine D2-R may lead to changes in AMPA-R trafficking that would support a decrease in synaptic strength we currently have been awarded an NIH grant that will begin to specifically address whether exercise induce neurophysiological and molecular changes in MSN AMPA-R expression is specific to D2 containing neurons. We have also

carried out neurophysiological studies to show that a DA-D2 receptor antagonists dampen the AMPA response within MSNs and again supporting the close interaction of these two neurotransmitter systems. In addition we have also submitted a NIH R21 that will begin to examine the mechanism(s) by which DA-D2 receptor may modulate AMPA-R trafficking through the targeting of Protein phosphatase 1 (PP1) and its scaffolding proteins Neurabin I and Neurabin II (Spinophilin)

***Study 5**: <u>We will test the hypothesis that exercise induced neuroplasticity can be attenuated through the administration of either a NMDA or AMPA receptor antagonist</u>. After MPTP-lesioning mice will be subjected to exercise while receiving either the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist GYKI-52466. Behavioral recovery will be compared between groups. Brain tissue will be analyzed for alteration in dopaminergic function (dopamine, DAT and TH expression). Pilot studies show that both glutamate receptor antagonists GYKI-52466 and MK-801 can be administered in this model of MPTP-lesioning*.

The primary goal of this study was to demonstrate using a pharmacological approach to delineate the relative contribution of either the NMDA or AMPA receptor subtype to behavioral recovery we observe in our model of basal ganglia injury. Findings from Study 4 of Specific Aim 1 using molecular biology approaches strongly suggested the AMPA-R GluR2 and its phosphorylated states as playing a major role. In light of this finding we took a more precise analysis incorporating in vitro slice cultures with pharmacological manipulation to test this model. For example, analysis of the relative contribution of NMDA to AMPA channels ratio were determined in the presence of NMDA antagonist (MK-801 or APV) or AMPA antagonist (GYKI-52466 or CNQX). Our findings showed an increase in the ratio of AMPA/NMDA currents after MPTP administration that decreased after exercise.

Other additions:

The Year Four figures showing 18F-fallypride binding potential changes in both the mouse and patients with Parkinson's disease undergoing intensive treadmill exercise are included as updated from additional data obtained in Year Five. Images are also included below.

As part of the studies in this grant we have also analyzed the pattern of expression of the DA-D2R using a number of complementary techniques including in situ hybridization histochemistry, western immunoblotting, and PET-Imaging. Presentation of findings from PET-imaging using the novel DA-D2R specific ligand is outlined in the next section. Our results as shown in the adjacent figure demonstrate elevated mRNA transcript for DA-D2R in what appear to be striatal medium spiny neurons as well as elevated striatal protein for DA-D2R with exercise in the MPTP-lesioned mouse model. The upper left panels show representative data of in situ hybridization histochemistry for all groups, which are graphically shown in the right panels. The lower left panels show results of western immunoblotting showing elevated DA-D2R protein expression with exercise in the MPTP-lesioned mouse model. Corresponding PET-imaging analysis is shown in the next section.

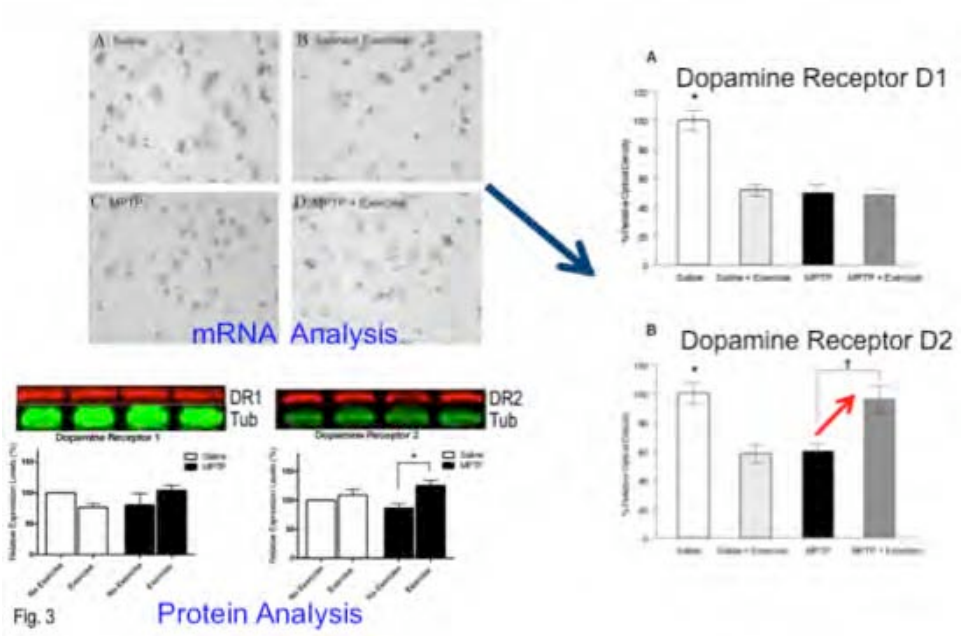


Fig. 3

Reportable Outcomes

Component 1: To test the hypothesis that exercise enhances neuroplasticity of the MPTP-lesioned mouse through glutamate by modulating dopamine biosynthesis.

Study 1*: The level of striatal dopamine and its metabolites will be determined using HPLC analysis comparing exercise versus non-exercise groups in the MPTP-lesioned mouse.*

This Aim has been completed. The findings are described in our recent publication Petzinger et al 2007 in *Journal of Neuroscience*. This manuscript is included in the Appendix of this report. Briefly, with exercise there is no enhanced return of striatal dopamine in the MPTP-lesioned mouse but rather we observed increased release of dopamine in surviving nigrostriatal dopaminergic projections with exercise. This analysis was made using fast-scan cyclic voltammetry.

There are no deviations from Study 1. However, to strengthen and further understand this phenomenon of alterations in dopamine with exercise, we have added two important studies: (i) fast-cyclic voltammetry; and (ii) accelerated mouse rotarod. The fast-scan cyclic voltammetry allowed us to discover that with exercise there is enhanced dopamine release in the MPTP-lesioned mouse model. These findings are described in Petzinger et al 2007, included in the Appendix of this report. The addition of the rotarod analysis of motor behavior allowed us to determine that there is in fact enhanced motor learning in the MPTP-lesioned mouse subjected to intensive treadmill exercise. These findings are also described in Petzinger et al 2007.

As a complement to extend these studies we have been developing a means to examine changes in dopamine neurotransmission in our model of exercise-enhanced motor behavior recovery. Since we observed changes in the dopamine D2 receptor using molecular techniques we wanted to know if similar changes could be detected in vivo in the mouse brain. Using PET-imaging with a novel dopamine D2-specific ligand 18F-fallypride we have shown that mice undergoing intensive treadmill exercise do in fact display increased expression of D2 as determined by increased binding potential for this PET imaging ligand.

Study 2*: The pattern of expression of striatal tyrosine hydroxylase (TH), dopamine transporter (DAT), cAMP-responsive enhancer binding protein (CREB), phospho~CREB, and dopamine- and adenosine- 3':5'-monophosphate-regulated phosphoprotein (DARPP-32), and phospho~DARPP-32 protein and their mRNA transcripts in surviving dopaminergic neurons will be determined using immunohistochemistry, western immunoblotting, in situ hybridization and correlated with striatal dopamine return. Pilot data shows attenuation of the return of DAT protein, and TH mRNA by exercise in MPTP-lesioned mice*.

We have completed the analysis of TH and DAT proteins using western immunoblotting and immunohistochemical staining, and the analysis of their mRNA transcripts using in situ hybridization histochemistry. These findings are described in Fisher et al 2004 and Petzinger et al 2007, both included in the Appendix of this report. The analysis of DARPP-32 and its phosphorylated isoform at Thr75 as well as CREB and its phosphorylated CREB is completed has been presented in abstract form and will be included in a manuscript to be prepared in the upcoming period.

There were no deviations from this aim.

Study 3: *The effect of exercise on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows altered glutamatergic synapses using immuno-electron microscopy*.

This aim has been completed. Results from this study using immuno-electron microscopy with an antibody against glutamate are presented in the published manuscript Fisher et al 2004 included as an Appendix in this report.

There were no deviations from this aim.

Study 4: *The pattern of expression of subunits for both the NMDA and AMPA receptor subtypes and their phosphorylated state will be determined using western immunoblotting, immunocytochemistry and in situ hybridization histochemistry*.

The first phase of this aim, analyzing the pattern of expression of the AMPA receptor subunits GluR1 and GluR2 using qRT-PCR and immunohistochemical staining within the dorsolateral striatum, has been completed. This analysis also included determination of the pattern of expression of the phosphorylated forms of these protein subunits as well as the expression of the flip and flop mRNA isoforms due to alternative splicing. These data are included in this report and are the basis of a manuscript vanLeeuwen et al 2008, included in the Appendix of this Report. We have also used qRT-PCR for the analysis of AMPA receptor subunits GluR2 and GluR3 as well as the NMDA receptor subunits NR1, NR2A through NR2D. We anticipate these findings to be included in a manuscript in the near future.

We have deviated from this aim with the addition of Golgi staining to determine potential alterations in the pattern of dendritic spine density. Our initial analysis included quantification of dendritic spine density within the dorsolateral striatum of mice from all four groups including (i) saline control, (ii) saline+exercise, (iii) MPTP-lesioned, and (iv) MPTP+exercise. The rationale for this approach is the fact that dendritic spine density is influenced by glutamatergic neurotransmission. We do observe MPTP-dependent changes comparing saline and MPTP-lesioned groups. We suspect that there are differences in the pattern of expression on D1-containing (direct path) and D2-containing (indirect path) medium spiny neurons but their discovery are masked by our inability to delineate between these striatal projection pathways using this approach since Golgi stain occurs in a subset of all neurons. Since we observe changes in dopamine receptor D2 with exercise (as outlined in Fisher et al 2004) we hypothesize that changes in dendritic spine density may be localized to this pathway. Therefore, we have obtained and have been breeding a transgenic strain of mice expressing green fluorescent protein in or D2-dependent striatal projection populations. This transgenic strain will play an important role in addressing the issue of morphological changes in specific striatal neuron populations.

We have deviated from this aim with the addition of quantitative real-time PCR using an Eppendorf realplex thermocycler recently acquired by our lab. This approach will allow for the analysis of mRNA transcripts to complement in situ studies and protein expression in our lesioning and exercise paradigm. Findings from this approach are included in this report and will be part of an upcoming manuscript to be submitted for publication. An advantage of this approach is that smaller amounts of starting tissue are required and the relative concentration in expression can be determined with high precision.

Findings that examine changes in the pattern of expression of AMPA receptor subunits GluR1 and GluR2 and their phosphorylated states have been submitted as a manuscript for publication. A copy in pdf form is included as an Appendix to this report.

Study 5*: We will test the hypothesis that exercise induced neuroplasticity can be attenuated through the administration of either a NMDA or AMPA receptor antagonist. After MPTP-lesioning mice will be subjected to exercise while receiving either the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist GYKI-52466. Behavioral recovery will be compared between groups. Brain tissue will be analyzed for alteration in dopaminergic function (dopamine, DAT and TH expression). Pilot studies show that both glutamate receptor antagonists GYKI-52466 and MK-801 can be administered in this model of MPTP-lesioning*.

The initial phase of this aim has been completed. We have administered both an AMPA receptor antagonist (GYKI-52466) and a NMDA receptor antagonist (MK-801) to mice administered either saline or MPTP. Findings suggest that the administration of glutamate antagonist attenuates the recovery of the nigrostriatal dopaminergic system following MPTP-lesioning. The next phase is to subject mice to these same glutamate antagonists while they are undergoing intensive treadmill exercise. At this point we intend to deviate from the initial experimental design in our approach but should reach the same scientific conclusions. In collaboration with Dr. John Wash (USC Andrus Gerontology Center) we have pursued the analysis of the glutamatergic contribution to exercise-enhanced neuroplasticity in striatal slice cultures in our model of

lesioning and exercise. Findings using fast-scan cyclic voltammetry show altered dopamine release in MPTP-lesioned mice subjected to intensive treadmill exercise have been included as an important part of our manuscript Petzinger et al 2007 in the Journal of Neuroscience (included in the Appendix). Studies currently underway include the examination of changes in the ration of AMPA to NMDA currents within the dorsolateral striatum, analysis of long-tern potentiation and long-term depression with exercise, as well as pharmacological examination of glutamatergic currents using subunit specific antagonists to delineate changes in channel composition in both NMDA and AMPA receptors. Studies examining changes in the AMPA to NMDA receptor currents with exercise are to be included in an upcoming manuscript.

Studies have been carried out using immuno-electron microscopy with an antibody against glutamate showing no significant alteration in glutamate synaptic occupancy in mice administered the AMPA receptor antagonists GYKI-52466 or the NMDA receptor antagonists MK-801.

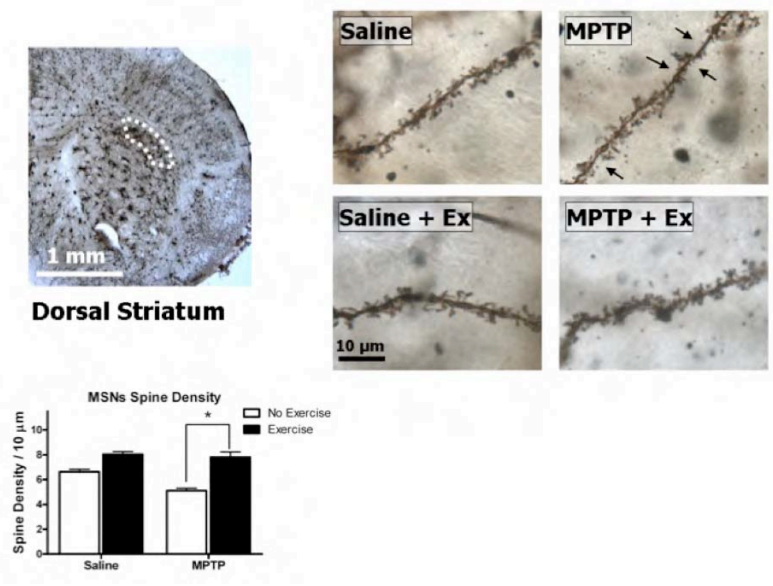
An objective of this Study is to better understand the relative contribution of the AMPA and NMDA receptors in the striatum following MPTP-lesioning and to determine if intensive treadmill exercise leads to meaningful changes that may underlie recovery of motor behavior. As a slight deviation from this Aim, which still addresses the core issues, has been the utilization of electrophysiological approaches to examine alterations in glutamate neurotransmission. As included in our manuscripts (Petzinger et al, 2007; vanLeeuwen et al, 2009) electrophysiological studies have supported our molecular findings that intensive treadmill exercise leads to increased expression of GluR2 (a subunit that leads to reduced calcium influx when incorporated into heteromeric glutamate channels) and changes in rectification consistent with this. Overall, the electrophysiological approaches we have employed allow us to better understand meaningful changes in the composition of glutamate receptors within the basal ganglia in our model of experience-dependent neuroplasticity. While pharmacological approaches selectively blocking AMPA or NMDA channels are one means to investigate glutamate receptor changes we have found that an approach using electrophysiological techniques has provide additional information that links molecular and physiological changes that may underlie the motor behavior recovery in our model.

To accomplish the goals of this study we decided to pursue experiments utilizing a similar pharmacological approach in an in vitro slice culture system with electrophysiological analysis. This approach avoided some of the potential confounders of systemic pharmacological treatment in mice that could interfere with motor behavior and balance in the motorized treadmill running task. The in vitro slice culture allowed direct comparison of the relative contribution of the AMPA or NMDA subtype of glutamate receptor within striatal MSNs in mice from all four groups (saline, saline+exercise, MPTP, and MPTP+exercise). Findings from these studies have supported the AMPA-R subunit GluR2 altered in its synaptic contribution (most likely due to altered trafficking and expression) and are included in published manuscripts (vanLeeuwen et al, 2009; Petzinger et al, 2009).

Additional studies completed in direct support of the Specific Aims of this grant and represented deviations from the initial experimental design.

(i) Quantification of dendritic spine density in the BAC-D2-eGFP transgenic mouse and determination of the relative contribution of the DA-D2R containing striatopallidal (indirect) pathway to exercise-dependent motor behavior recovery.

Findings from studies in this grant indicated elevated expression of both DA-D2R and the AMPA-R GluR2. Since dendritic spine morphology is closely related to glutamatergic neurotransmission within synapses and that the DA-D2R is localized predominantly to the striatopallidal indirect pathway we speculate that there are alterations in spine morphology specific to the indirect pathway. To delineate between the DA-D1R containing direct pathway and the DA-D1R containing indirect pathway we have obtained a transgenic mouse strain termed BAC-D2-eGFP in which the green fluorescent protein is expressed exclusively within DA-D2R containing indirect projection neurons. Baseline studies have established that these mice are susceptible to MPTP similar to standard C57BL/6 mice based on striatal dopamine depletion, loss of striatal tyrosine hydroxylase protein, and unbiased stereological counting of nigrostriatal dopaminergic neurons. We have established a colony of these transgenic mice and have breed sufficient numbers of male mice to allow for studies involving saline, saline+exercise, MPTP, and MPTP+exercise groups. Based on the fluorescence within the indirect pathway projection neurons we can distinguish the indirect (green) from the direct (no green) pathway neurons. Studies are now underway to evaluate the relative changes we observe in AMPA-R GluR2 specifically to indirect pathway neurons based on both immunohistochemical staining for protein and electrophysiological properties through single-cell recording. Cells in which we have recorded from are injected with biocytin, processed immunohistochemically, and the density of dendritic spines determined by light microscopy and computer assisted image analysis. These studies are now supported by a current NIH RO1 grant.



The Figure to the left demonstrates analysis of Golgi stained MSNs within the dorsolateral striatum showing increased spine density with exercise in MPTP-lesioned mice. These findings are the subject of a manuscript in the latter stages of preparation (Vuckovic et al, 2009).

(ii) Selective targeting of proteins involved in AMPA-R trafficking.

One potential mechanism by which AMPA-R subunit GluR2 and its phosphorylated states can change synaptic occupancy, as shown by studies in this grant, is by altered receptor trafficking from extra-synaptic to synaptic sites. Based on reports in the literature we hypothesize that two proteins may be potentially important for AMPA-R trafficking; these include the proteins neurabin I and spinophilin (also called neurabin II). To test their potential role in receptor trafficking we have developed, in collaboration with the laboratory of Dr. Pin Wang (Bioengineering, USC) we have constructed two lentivirus based vectors carrying siRNA specific sequences designed to knock-down neurabin I and spinophilin in vivo. At the writing of this report we have completed the following: (i) construction of lentivirus vectors carrying siRNA and red fluorescent proteins, (ii) validation of protein expression target knock-down in cell culture, and (iii) delivery of both vectors to the striatum of BAC-D2-eGFP mice to demonstrate targeting and infectivity to medium spiny neurons. The strategy we have developed is to deliver the red fluorescent protein vector to green cells of the indirect pathway such that those cells of the indirect pathway (expressing GFP) will fluoresce yellow (green plus red) while those cells of the direct pathway will fluoresce red only. This strategy will be utilized to specifically examine the consequences of knock-down of expression of neurabin or spinophilin on the trafficking of GluR2 into synapses with electrophysiology (analysis of rectification, EPSCs and AMPA/NMDA ratios), molecular (immunohistochemical analysis of protein expression), and dendritic spine morphology. These studies are now included in an R21 application to the NIH in its second submission following an initial score in the 50th percentile. With our additional preliminary data including vector characterization and validation of the approach we are confident the current submission will be met with increased enthusiasm.

The Next page has a summary of figures demonstrating this experimental approach.

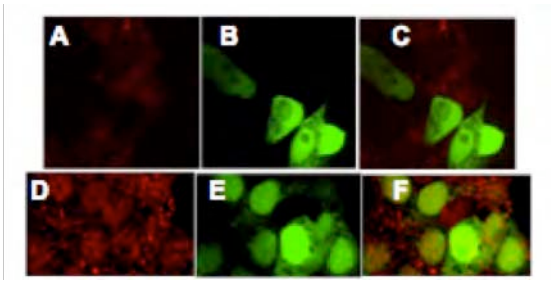


Figure 1. Knockdown of Neurabin in 293T cells: 293T cells were transfected with a lentivector expressing eGFP + shRNAmir construct FUGW-VSVG (green) which targets Neurabin. Cells were seeded with non-transduced 293T and immunostained with Neurabin antibodies (red). A though C) Neurabin knockdown. Arrow indicates absence of Neurabin protein due to knockdown. D through F) Control experiments showing cells transfected with eGFP only and immunostained with Neurabin antibodies. Arrow indicates neurabin co-localization with eGFP.

Figure 2. Neurabin Knock Down In Vitro: 293T cells were employed to test the effect of eGFP+shRNAmir 73 FUGW-VSVG lentivector transfection. Western Blot analysis shows a marked reduction in eGFP+shRNAmir 73 transfected cells compared to control 293T cells and eGFP-only transfection. β actin served as control.

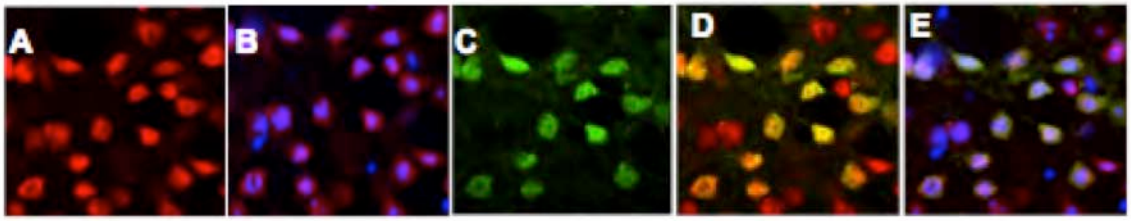


Figure 3. shRNAmir construct + eGFP In Vivo Transfection in Medium Spiny Neurons: High magnification confocal images of medium spiny neurons from a mouse striatal slice. The FUGW-VSVG lentivector specifically knocks down the actin-binding protein neurabin. The lentivector was delivered stereotaxically to the dorsolateral striatum at Bregma coordinates (1.00, 1.00 lateral, 2.00 below dura). Brains were harvested two weeks after delivery. (A) Immunohistochemistry with neuronal marker NeuN in red. (B) Merged immunostaining for NeuN (red) and TOPRO3-Cy5 a DNA marker (blue). (C) FUGW-VSVG lentivector that expresses eGFP (green) under control of the human ubiquitin C promoter. (D) Merged image NeuN and FUGW-VSVG lentivector expressing eGFP (green). (E) Merged image NeuN and FUGW-VSVG lentivector expressing eGFP (green) and TOPRO3-Cy5 (blue). Untransfected sections do not show eGFP expression. This figure supports Aim 1 by confirming that stereotaxic delivery is an effective means to target medium spiny neurons in an In Vivo system. Further research utilizing electrophysiological measures will allow us to investigate the role that knocking down neurabin plays in synaptic strength.

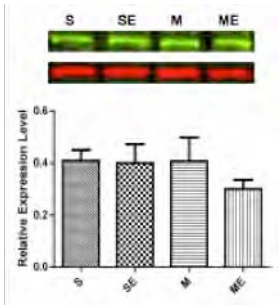
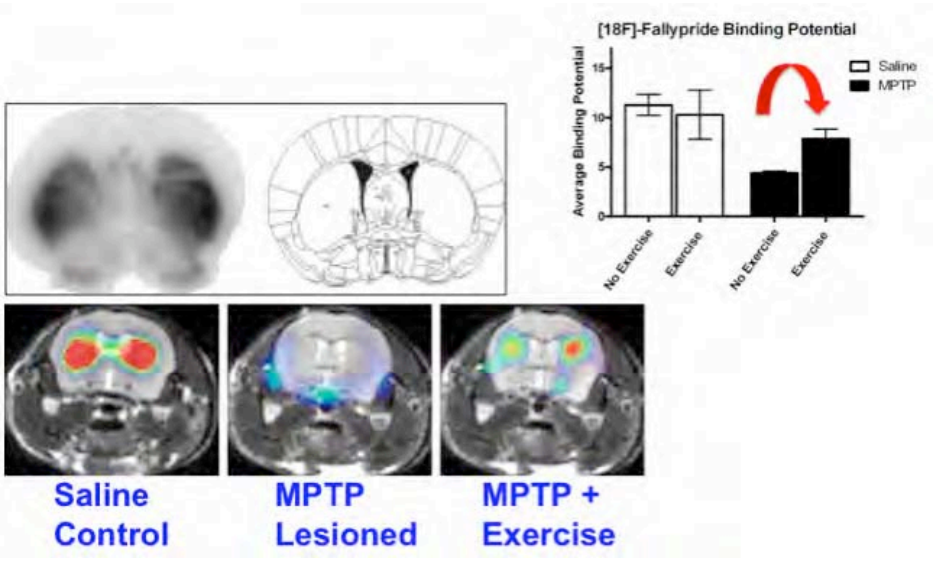


Figure 4. Neurabin Total Protein Expression: Western Blot analysis in total tissue homogenates of mouse dorsolateral striatum in groups Saline (S), Saline Exercise (SE), MPTP (M), and MPTP Exercise (ME). Neurabin (NRB) optical density values were normalized against Tubulin (T). No statistically significant differences were observed in the mean relative expression levels across the four groups at $\alpha < 0.05$. This figure support Aim 2 by showing that neither MPTP nor Exercise has an effect on the expression of Neurabin before knock down via shRNA lentivector delivery.

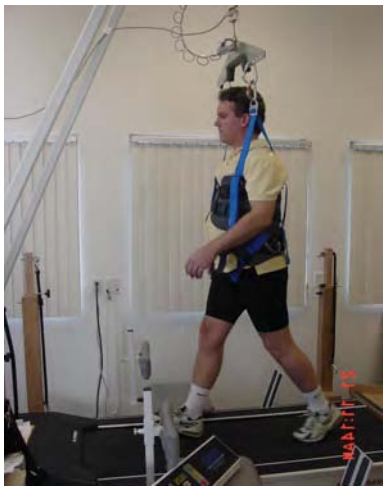
(iii) Analysis of the expression of the DA-D2R in the MPTP mouse with exercise using PET-imaging with the DA-D2R specific ligand 18F-fallypride.



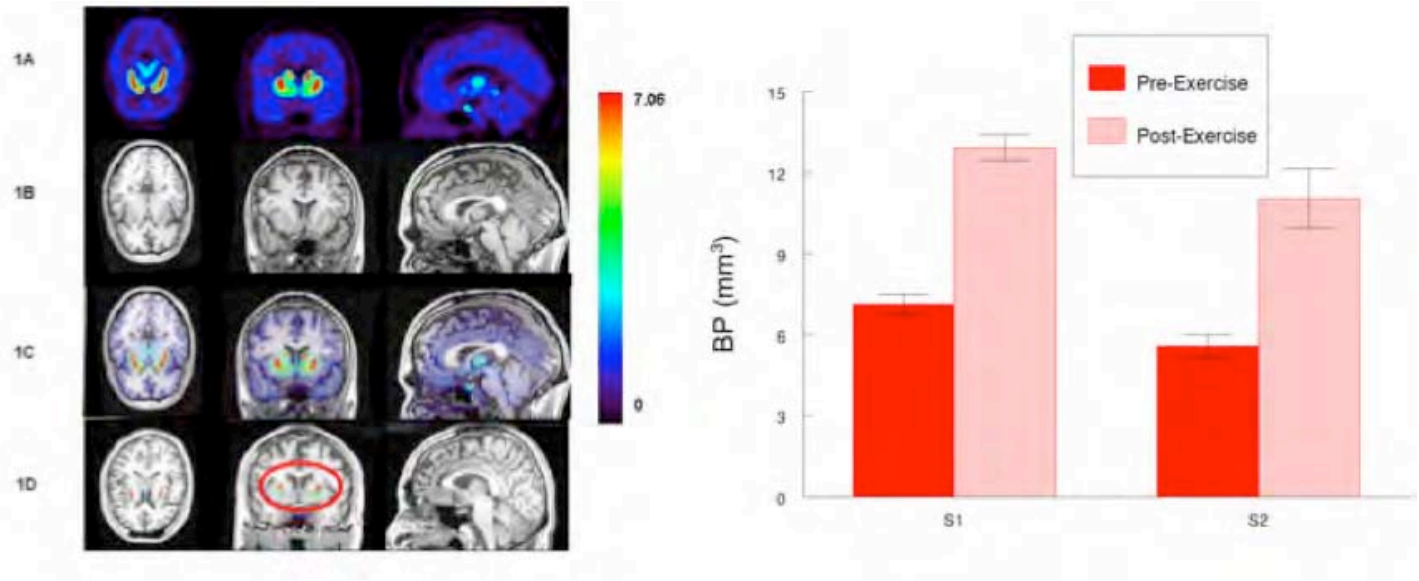
Based on our molecular studies in this grant we have demonstrated elevated expression of DA-D2R using protein analysis with immunohistochemistry and western immunoblotting and analysis of mRNA transcript using in situ hybridization histochemistry. An important step to develop translational clinical studies in patients with Parkinson's disease based on these findings is to demonstrate and validate a non-invasive in vivo approach. We have selected PET-imaging utilizing the novel DA-D2R specific ligand 18F-fallypride. Basically, mice from all four groups including saline, saline+exercise, MPTP, and MPTP+exercise groups (n = 6 to 8 per group) were subjected to PET-imaging prior to the start of the exercise intervention. Exercise groups were exercised using our established protocol of one hour per day for a total of 28 running days (5 days per week). At the end of the exercise regimen as well as 6 weeks after exercise completion mice from all groups were subjected to PET-imaging again. Our findings indicate that MPTP-lesioned mice exposed to intensive treadmill exercise, but not saline treated mice, show a significant increase in 18F-fallypride binding potential consistent with elevated expression of DA-D2 receptor numbers. These results are included in a manuscript now in the final stages of preparation (Vuckovic et al, 2009).

(iv) Analysis of the expression of the DA-D2R using PET-imaging with the DA-D2R specific ligand 18F-fallypride in patients newly diagnosed with Parkinson's disease undergoing body-weight supported treadmill training.

In vitro and in vivo studies in mice demonstrate increased expression of the DA-D2R with intensive treadmill exercise following MPTP administration. A major goal of our research program is to translate findings from the laboratory to clinical studies in patients with Parkinson's disease to develop new therapeutic treatment modalities. In the final year of this grant, while not part of the initial Specific Aims, we have carried out a pilot study to demonstrate the feasibility and efficacy of PET-imaging with treadmill running in patients with Parkinson's disease. Newly diagnosed patients (within one year of diagnosis; n = 4) were subjected to either body-weight supported treadmill running (3 x 45 minute sessions per week for 8 weeks) or no exercise. Age matched controls (n = 4) were also subject to treadmill running or no exercise. Subjects form all groups underwent PET-imaging with 18F-fallypride prior to the exercise period and at completion of the exercise regimen. The two patients with Parkinson's disease undergoing bodyweight supported treadmill exercise both showed increased 18F-fallypride binding potential of 90 to 95% over pre-exercise baseline levels. Patients in the no exercise group did not show a significant change in ligand binding potential. The image to the left shows an example of the body-weight supported treadmill training apparatus. Details of the use of this apparatus in our research program can be found also in published manuscripts including Fisher et al, 2008.



This figure demonstrates findings utilizing the DA-D2R specific ligand 18F-fallypride in PET-imaging in newly diagnosed patients with Parkinson's disease. The left panel shows row 1 images from 18F-fallypride PET-imaging, row 2 corresponding MRI, row 3 co-registration of PET-images and MRI, row 4 subtraction of pre-exercise and post-exercise PET-images with MRI co-localization. The circled region of interest in row 4 middle coronal image shows elevated 18F-fallypride binding potential consistent with elevated DA-D2R binding. The data on the right panels shows quantification of pre- and post-exercise PET-images from 2 subjects. These patients were within one year of diagnosis for Parkinson's disease and were not on any drug treatment. These data are the direct result of studies supported through this grant and have now formed the basis of translational studies that are ongoing as well as studies that are the primary focus of a clinical trail included in an RO1 application submitted to the NIH NINDS in July 2009.



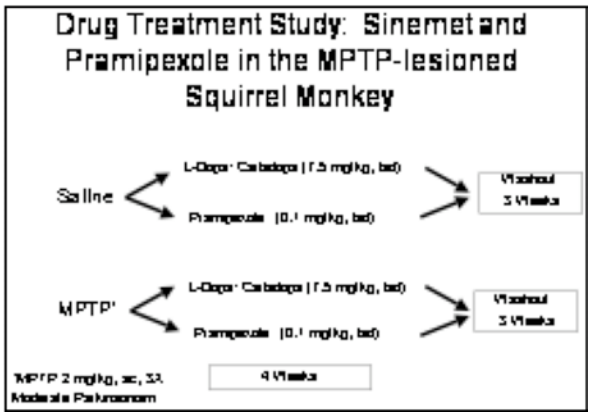
Overall Impact of findings from Component 1

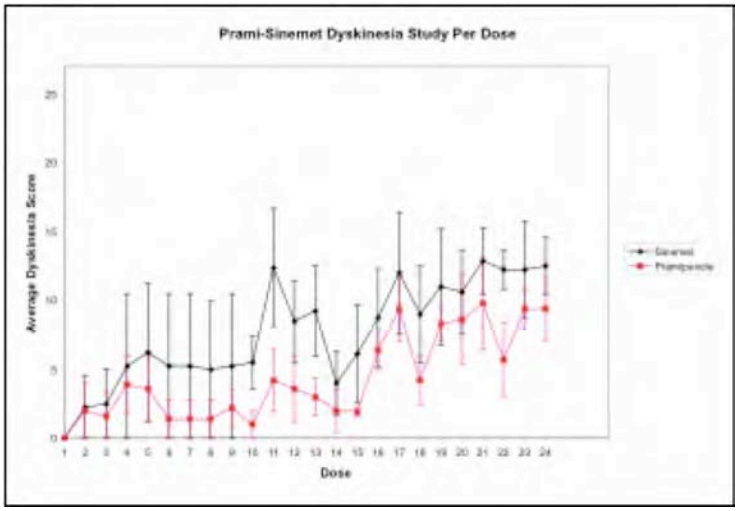
The most significant impact of studies supported in this research grant is the translation of findings from the mouse model of Parkinson's disease to studies in patients with Parkinson's disease. Briefly, analysis of the dopaminergic system in mice undergoing intensive treadmill exercise and showing benefit in motor behavior displayed elevated expression of DA-D2R within striatal medium spiny neurons. In vivo studies, also in the rodent model, using the DA-D2R specific ligand 18F-fallypride with PET-imaging also supported changes in DA-D2R with exercise in the injured brain. Translational studies carried out in newly diagnosed patients with Parkinson's disease also using 18F-fallypride also showed elevated DA-D2R binding in those patients undergoing treadmill running, but is not seen in sedentary patients. Studies are ongoing to determine the effect of exercise in normal age-matched controls undergoing treadmill exercise. Taken together studies supported from this grant demonstrate the value of the animal model in developing hypothesis driven clinical studies, and provide a means to investigate the underlying mechanism of exercise-dependent changes in DA-D2R seen in patients. Importantly animal studies supported by this grant have provided important insight and rationale regarding the importance of exercise induced changes in the DA- D2R expression in driving "normal" synaptic connections within the basal ganglia of individuals with Parkinson's Disease. Specifically our animal studies have supported the importance of the AMPA-R in exercise-induced neuroplasticity and the potential role of the DA-D2 receptor in modulating this trafficking. Findings from this study are having a direct and immediate impact on patient care in that it is providing important justification for prescribing intensive exercise as soon as an individual is diagnosed with Parkinson's disease in order to enhance the dopaminergic signaling and to support synaptic connections within the basal ganglia. This work has also provided critical scientific rationale to begin a multicenter study examining the effects of intensive exercise in modifying the course of Parkinson's disease. Investigators on this grant are key investigators in this multicenter human exercise study that will be submitted as an RO1 in February of 2010.

Component 2: Pharmacological Enhancement of Neuroplasticity in the MPTP-lesioned Non-Human Primate Model.

Study 1: *The behavioral recovery of saline injected and MPTP-lesioned squirrel monkeys will be compared with and without the administration of Pramipexole. Animal behavior will be monitored using both a cage side clinical rating scale and a personal activity monitor.*

An additional phase of this aim was to characterize the behavioral, morphological, and neurochemical neuroplasticity (recovery) in the MPTP-lesioned nonhuman primate without pharmacological treatment. The analysis of TH, DAT, midbrain dopaminergic neurons, motor behavior, and levels of CPu dopamine were evaluated in squirrel monkeys administered MPTP in a series of 6 or 2 injections (subcutaneous, 2 mg/kg free-base, 2 weeks between injections) over several months. Six injections corresponded to a moderate parkinsonian state and two injections to a mild parkinsonian state. These findings are described in detail in a recently published manuscript Petzinger et al, 2006, which is included in the appendix of this report. One interesting finding of this study was that the return of normal motor behavior at 9 months in MPTP-lesioned squirrel monkeys rendered moderately parkinsonian was accompanied by an incomplete return of striatal dopamine. Specifically we observed a greater than 90% level of total dopamine depletion in the dorsolateral putamen at 9 months when animals were fully recovered. While it has been reported that pre-synaptic adaptations in remaining dopaminergic neurons and terminals are thought to lead to normalization of extracellular levels of dopamine especially in animals with lesions resulting in less than 80% depletion, it has also been shown that animals with greater than 80% depletion show only partial normalization of extracellular striatal dopamine (Castaneda 1990). We carried out voltammetry studies in a subset of moderately parkinsonian animals at 9 months post-MPTP lesioning, to examine changes within the nigrostriatal terminals. We observed that the dopaminergic terminals in the dorsolateral striatum remained substantially depleted (> 90%) (see Study 4 below) along with the total levels of striatal dopamine. As other investigators have reported, however, we observed was a greater degree of total dopamine return in the ventral striatum and a normalization of dopamine turnover. Tyrosine hydroxylase and DAT expression was increased in late stage recovery even in dopamine depleted regions and supports a role for sprouting. We also observed an increase in DARPP-32 expression within medium spiny neurons of recovered animals, which supports the role of post-synaptic compensatory changes as an underlying mechanism of this recovery.

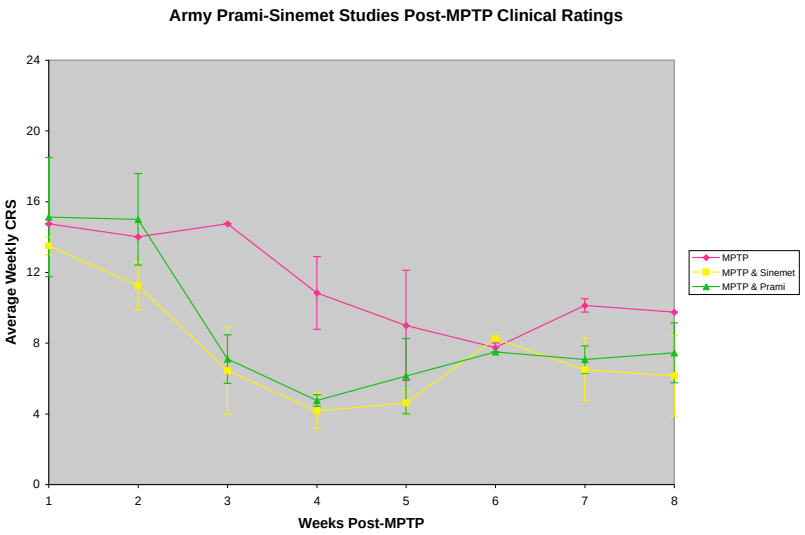




The main component of aim 1 was to investigate the long-term effect of dopamine replacement therapy on the behavioral recovery of the MPTP-lesioned squirrel monkey. For study 1 we treated MPTP-nonhuman primates with either Pramipexole or L-DOPA/ Carbidopa for 4 weeks (3 days/week, twice daily, Tues-Thurs) and then washed out for 3 weeks. A baseline evaluation was performed on week 1, animals were treated with dopamine replacement therapy from week 2-5, and then animals were washed out weeks 6-8. We added a Sinemet (L-DOPA/ Carbidopa) group for comparison with Pramipexole based on the scientific rationale that L-dopa, unlike Pramipexole, is metabolized and stored by dopaminergic terminals and therefore may have a more direct effect on the regulation of endogenous dopamine production and behavioral recovery and offers an interesting comparison to a compound that is not taken up by terminals.

One important outcome in Study 1 was the unexpected induction of dyskinesia in the MPTP-lesioned animals administered Pramipexole. This new finding has not been reported in the literature by other investigators and we are preparing a manuscript reporting this novel finding (Figure 8). This may indicate that Sinemet and Pramipexole may both induce dyskinesias through similar mechanisms.

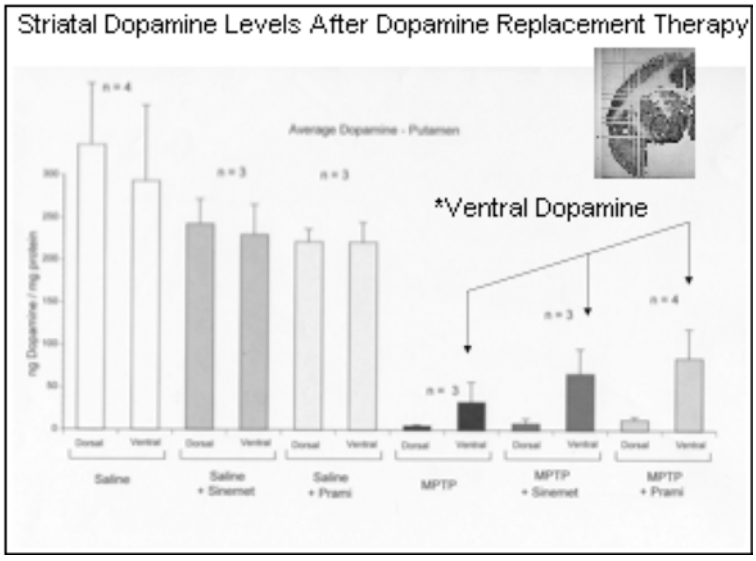
The behavioral assessment in this first group of animals carried out up to 8 weeks after the last injection of MPTP showed a slight enhancement of behavioral recovery in both the Sinemet and Pramipexole groups versus the saline treated group (Figure 9).



Nonhuman primates that were initially to be used for the long-term behavioral studies, were used for (1) adding a L-dopa group (2) conducting microdialysis studies to extend our findings in Study 2 (neurochemistry); and (3) to examine alterations in electrophysiolgical properties of corticostriatal neurons and voltammetry. See Sections below.

***Study 2**: Analysis of brain tissue from MPTP-lesioned squirrel monkeys administered Pramipexole or L-dopa/carbidopa or saline. This analysis included neurochemistry and molecular studies that examined the pattern of expression of proteins and mRNA transcripts important for dopaminergic function (including TH, DAT, VMAT2) at the level of the SNpc and CPu.*

Studies under this aim included (1) HPLC analysis of dopamine and its metabolites in the dorsal and ventral caudate nucleus and putamen, (2) Microdialysis of dopamine in the putamen, and (3) western immunoblot analysis of CPu proteins including TH, DAT, VMAT-2, DARPP-32, DARPP-32~phosphoThr34, and DARPP-32~phosphoThr75. Immunohistochemical staining for these same proteins are currently underway and their analysis is not yet completed. The following section highlights our current findings using these techniques for Study 2.

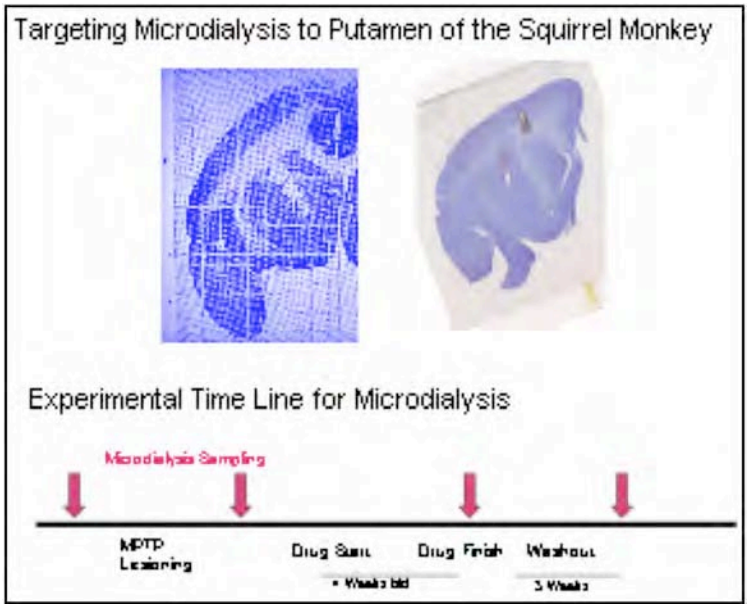


(1) Total dopamine levels and metabolites were analyzed from all groups of animals, using HPLC. Brains were removed from all groups at completion of 4 weeks of drug or saline treatment, followed by 3 weeks of drug washout. Microdialysis of the putamen was added to this study to complement and support findings from out HPLC analysis of tissue. We found there was a slight increase in dopamine levels in the ventral caudate and putamen of animals receiving Pramipexole or Sinemet. These results are shown in Figure to the left.

Figure: Analysis of dopamine and its metabolites in the MPTP-lesioned squirrel monkey. Squirrel monkeys were MPTP-lesioned and then treated one week after the last injection of MPTP with

either Sinemet (10 mg/kg twice daily), or Pramipexole (1 mg/kg twice daily). Animals were treated for four weeks. On each week animals received drug for three days (Tue, Wed, Thurs)) and then saline for four days (Fri, Sat, Sun, Mon). Animals were rated each day for parkinsonian features and for dyskinesia. Drug was washed out for 3 weeks and then animals were euthanized. Brain tissue was collected and striatal tissue dissected 8 weeks (1 week monitoring + 4 weeks drug treatment + 3 weeks washout) after MPTP. HPLC analysis showed that Pramipexole and Sinemet (L-dopa + carbidopa) treated animals had a slight increase in striatal dopamine, especially in the ventral putamen, compared to MPTP + saline treated nonhuman primates.

(2) Microdialysis was carried out to determine in vivo levels of dopamine within the putamen. Briefly, microdialysis was carried out on 3 squirrel monkeys prior to MPTP-lesioning (as baseline), immediately following MPTP lesioning, during treatment with either pramipexole or Sinemet, and again after a 3-week washout. The primary finding was that pramipexole treated animals displayed elevated amphetamine-evoked dopamine release compared to MPTP untreated animals. Sinemet animals had a level of dopamine release intermediate to these two groups. The adjacent Figure shows the timeline of microdialysis studies. Figure 12 shows HPLC analysis of dopamine levels from microdialysis.



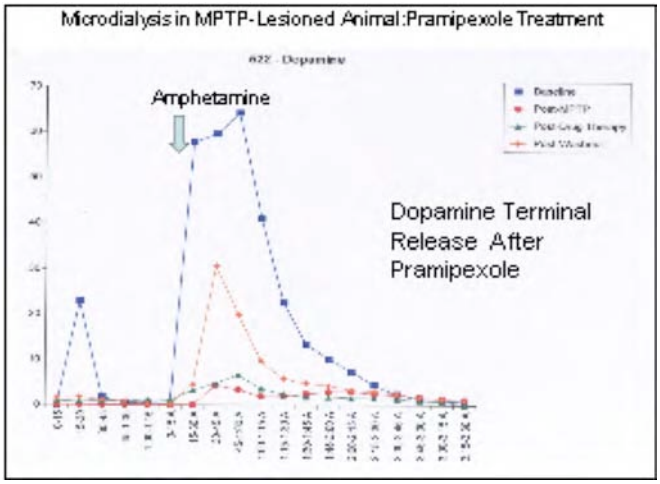
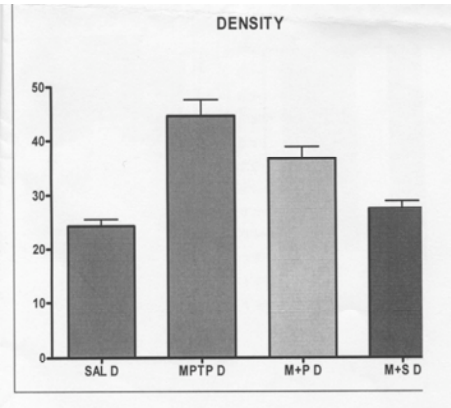


Figure to the left shows a representative microdialysis experiment with the same animal used as its own control and undergoing repeated microdialysis studies. Our studies show that Pramipexole or Sinemet treated animals have greater amphetamine-induced dopamine release.



(3) Western immunoblot analysis of proteins for TH, DAT, VMAT-2, and DARPP32 were carried out on tissues derived from either the ventral or dorsal caudate nucleus or ventral or dorsal putamen. Figure 13 displays a subset of the analysis of the western immunoblot data. To summarize, we found that within the dorsal caudate and putamen there is a slight elevation of TH and DAT expression in MPTP-lesioned animals treated with either Sinemet or pramipexole. There was no apparent change in VMAT-2 expression between all lesioned groups. There appears to be elevated DARPP-32 expression in MPTP-lesioned animals treated with Sinemet. There was also an increase in the phosphorylated forms of DARPP-32 (~phosphoThr34, and ~phosphoThr75) in Sinemet treated animals that was not observed in Pramipexole treated animals. We are currently analyzing the remaining western immunoblots and determining if our findings are consistent amongst the groups. We are also finishing the western blot analysis of the ventral caudate and putamen. These analyses plus the determination of the profiles of expression of the dopamine receptors will be completed in year 4 of this proposal. These studies will be complemented with immunohistochemical staining of tissues sections with antibody probes against TH, DAT, DARPP-32, and VMAT-2. Staining of sections has just been completed and is currently under analysis.

Much of the preliminary data generated in this Aim using western immunoblotting approaches were presented in the previous Annual Report. The purpose of the no-cost extension is to permit completion of these studies. We anticipate that this Aim will be completed in the final phase of this grant.

Study 3: *The pattern of expression of the dopamine receptors D1, D2, and D3 will be determined in both the SNpc and CPu. The level of protein expression will be determined western immunoblotting, immunohistochemistry, while the level of mRNA transcript expression will be determined using in situ hybridization histochemistry. Double labeling techniques will be used to co-localize the dopamine receptor changes with other enkephalin or substance P containing neurons. Preliminary data supports our ability to use these techniques in the non-human primate*.

This aim will be carried out in the final phase of this grant as part of the request for a no-cost extension.

Study 4: *The effect of Pramipexole on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows our ability to quantify glutamatergic synapses using immuno-electron microscopy.*

In collaboration with Dr. Charles Meshul (Oregon Health Sciences University, Portland, OR) perfusion fixed brain tissues were harvested from a nonhuman primate from each group for analysis using immuno-electron microscopy with an antibody against glutamate. These results are summarized in the Figure below. Following MPTP-lesioning there is an increase in the relative density of striatal glutamate immunolabeling (second bar) within corticostriatal terminals. After treating MPTP-lesioned animals with Pramipexole or Sinemet the relative density of glutamate immunolabeling is reduced. Increased density of striatal glutamate within the terminal is thought to reflect decreased glutamate release. Our study would suggest that Sinemet increases glutamate release to a slightly greater extent than Pramipexole. This increased glutamate release may be one means by which dyskinesia is elicited to a greater extent in Sinemet treated animals than Pramipexole treated animals.

The following studies have been added to this proposal with the aim of understanding the impact of alterations in the expression of glutamate receptors on the electrophysiological properties of striatal neurons.

Electrophysiological Studies of Basal Ganglia Function the Nonhuman Primate Model of PD

An important aspect of the nonhuman primate is the anatomical similarity of basal ganglia structure and function to that of humans, thereby providing an important tool for investigating basal ganglia function, such as neurophysiological properties in the normal and disease state, and thus serves as the foundation for identifying new therapeutic treatments. For example neurophysiological studies have implicated over-activity at corticostriatal synapses as one underlying mechanism for the development of motor impairment in PD (Konitsiotis *et al.*, 2000; Soares *et al.*, 2004; Wichmann and DeLong, 2003). Electrophysiological studies in our labs, using the MPTP-lesioned squirrel monkey, have shown changes in AMPA and GABA mediated synaptic neurotransmission that may account for excessive excitatory corticostriatal drive. For these studies, we administered MPTP in a series of 6 subcutaneous injections of 2.0 mg/kg (free-base) every 2 weeks for a total of 12 mg/kg. Whole brains were harvested at either 6 weeks (when animals are parkinsonian) or 9 months (when animals are motorically recovered) after the last injection of MPTP and striatal synaptic function was examined in coronal *in vitro* brain slices. We found that the input/output relationship was greater for AMPA receptor mediated synaptic currents at 6 weeks after MPTP-lesioning compared to saline control using whole cell voltage clamp. The relative strength of $GABA_A$ versus AMPA receptor mediated synaptic responses was calculated as the I_{GABA-} changes in $_A$ / I_{AMPA} ratio. Interestingly, we also found a reduced I_{GABA-A}/I_{AMPA} ratio 6 weeks after MPTP. These GABAergic inhibition that we and others have observed may play an important role in facilitating the synchrony and oscillatory patterns of discharge found throughout the basal ganglia motor circuit in MPTP-treated akinetic primates (Goldberg *et al.*, 2002; Raz *et al.*, 1996; Raz *et al.*, 2001). Analysis of animals 9 months after MPTP administration suggests there is normalization of corticostriatal hyperactivity when animals demonstrate full behavioral recovery. Specifically we found the input/output ratio for AMPA receptor-mediated synaptic responses and the I_{GABA-A} / I_{AMPA} ratio returned back to control levels (Figure 3). These observations are in agreement with the view that excessive glutamatergic corticostriatal synaptic function may be a contributing factor to the behavioral pathology of PD (Konitsiotis *et al.*, 2000; Muriel *et al.*, 2001). Future studies will exam whether changes in glutamatergic drive in fully recovered animals differentially impacts corticostriatal synapses in direct versus indirect basal ganglia pathways, as has been reported in the parkinsonian state (Day *et al.*, 2006; Wichmann and DeLong, 2003).

Dopamine denervation in animal models of PD is also associated with changes in the molecular composition of AMPA and NMDA receptors in the striatum (Betarbet *et al.*, 2004; Betarbet *et al.*, 2000; Hallett *et al.*, 2005; Hurley *et al.*, 2005; Nash *et al.*, 2004). We also found evidence for changes in the pharmacological profile of AMPA and NMDA receptors, which are consistent with these molecular studies. For example as shown in Figure 3, in animals examined 6 weeks post MPTP-lesioning, we found; (i) a decrease in the I_{NMDA}/I_{AMPA} ratio; (ii) an alteration in the NMDA receptor subunit composition as indicated by increased sensitivity to the selective NR2B antagonist CP-101,606; and (iii) an alteration in AMPA receptor mediated synaptic responses, as indicated by changes in the sensitivity to the selective AMPA receptor antagonist, GYKI-52466 compared to saline control animals (Nash *et al*., 2004; Ruel *et al.*, 2002). Again, with behavioral recovery at 9 months post-MPTP-lesioning, we observed the trend of a return of NMDA and AMPA receptor function to match that seen in saline injected squirrel monkeys (Figure 14).

The glutamatergic corticostriatal and the dopaminergic nigrostriatal system are important mediators of synaptic plasticity, termed long-term depression (LTD) and long-term potentiation (LTP), within the basal ganglia (Centonze *et al.*, 2001; Mahon *et al.*, 2004; Picconi *et al.*, 2005; Reynolds and Wickens, 2002). Electrophysiological studies in our lab, using saline control squirrel monkeys, have shown that the induction of long-term synaptic plasticity at corticostriatal synapses is region specific, with LTP being induced in more medial regions and LTD in more lateral regions. These findings agree with previous reports from the rodent model of PD (Partridge *et al.*, 2000; Smith *et al.*, 2001). Studies in the rat have shown a loss of synaptic plasticity after 6-OHDA administration, which we have observed in the MPTP-lesioned mouse model, 1 to 2 weeks after neurotoxicant exposure (Calabresi *et al.*, 1992; Centonze *et al.*, 1999; Kreitzer and Malenka, 2007). Presently, there is little known regarding alterations in synaptic plasticity immediately following MPTP-lesioning in the nonhuman primate.

Analysis of the expression of synaptic plasticity in the squirrel monkey 9 months after MPTP-lesioning has shown that LTD and LTP expression is evident. In the same animals used for analysis of glutamate neurotransmission above, we observed a dramatic and permanent decrease in dopamine release as measured by fast-scan cyclic voltammetry (Cragg, 2003) (Figure 4). This finding is in agreement with previous reports

examining dopamine function in the squirrel monkey using HPLC (Petzinger *et al*., 2006). The expression of dopamine-dependent forms of LTP we observed in the dopamine depleted squirrel monkey suggest an adaptation may occur in the expression and/or sensitivity of both D1 and D2 receptors (Centonze *et al*., 2001; Mahon *et al*., 2004; Picconi *et al.*, 2005; Reynolds and Wickens, 2002). Preliminary studies in our lab have shown that LTD expression at lateral cortico-putamen synapses from the 9-month MPTP-lesioned squirrel monkey is D2 dependent, since this effect is blocked by the D2 receptor antagonist *l*-sulpiride. In addition, use of *l*-sulpiride results in the unexpected expression of LTP in lateral synapses (Figure 4). Our findings are consistent with the literature, where dopamine receptors D1 and D2 have been shown to play an important role in LTP and LTD, respectively (Calabresi *et al.,* 1992; Centonze *et al.*, 1999; Wang *et al.,* 2006). Taken together, these data suggest behavioral recovery from MPTP exposure in the squirrel monkey may be due at least in part to compensatory increases in the sensitivity of dopamine receptors, which enables the normal and expected expression of long-term plasticity at corticostriatal synapses.

Conclusions:

The MPTP-lesioned mouse and squirrel monkey are valuable models for investigating neuroplasticity of the injured basal ganglia. These models can serve as valuable tool to investigate the molecular mechanisms by which extrinsic factors can be applied to enhance recovery. In mice, studies in this proposal are designed to determine the role of intensive treadmill exercise in enhancing motor recovery. Meanwhile the nonhuman primate, with its exquisite parkinsonian features and similarity of anatomical features to the human condition, serves as an excellent means to examine the role of pharmacological replacement therapy targeting the dopaminergic system and the potential role in influencing recovery.

Studies in the MPTP-lesioned mouse model and exercise from the first component of this proposal indicate that intensive treadmill exercise can enhance motor behavioral recovery employing mechanisms that are different from those seen with intrinsic neuroplasticity. Our results indicate that intensive treadmill exercise in MPTP-lesioned mice leads to (i) increased motor recovery and enhanced motor learning (ii) suppression of striatal DAT and TH proteins, (iii) increased stimulus evoked dopamine release as seen in fast-scan cyclic voltammetry, (iv) differential expression of DAT and TH mRNA transcripts, (v) altered expression of specific subunits of the AMPA and NMDA receptor subtypes, and (vi) altered expression of the dopamine receptor D2.

Studies in the second component of this proposal utilizing the MPTP-lesioned nonhuman primate show that the pharmacological application through dopamine replacement therapy with Sinemet (levodopa plus carbidopa) or the D2/D3 agonist pramipexole leads to enhancement of the intrinsic neuroplasticity we observe. For example, treated MPTP-lesioned animals show (i) increased levels of striatal dopamine, (ii) increased amphetamine-evoked dopamine release using microdialysis, (iii) elevated levels of TH and DAT protein in the caudate and putamen, (iv) differential expression of DARPP-32 and its phosphorylated forms in the caudate-putamen. Electrophysiological studies have shown a shift in the AMPA/NMDA ratio, altered corticostriatal drive, shifts in the subunit composition of glutamate channels, and that LTD expression at lateral cortico-putamen synapses from the 9-month MPTP-lesioned squirrel monkey is D2 dependent. One unexpected finding was the development of dyskinesia during treatment with Pramipexole, a behavioral characteristic not yet reported in the scientific literature.

Reportable Outcomes For Years One to Five

The following sections outline the reportable outcomes including Abstracts, Manuscripts, and Presentations. The manuscripts are included as pdf attachments.

Abstracts:

(1) Society for Neuroscience Annual Meeting, San Diego, 2004 ABSTRACT #1

Behavioral recovery in the MPTP-lesioned nonhuman primate: Altered dopamine biosynthesis and storage.
Hogg, E, M. W. Jakowec, K. L. Nixon, A. T. Abernathy, P. Arevalo, B. E. Fisher, M. Liker, and G. M. Petzinger.

(2) Society for Neuroscience Annual Meeting, Atlanta 2006 ABSTRACT #1

Exercise induced behavioral recovery and plasticity in the MPTP-mouse model of Parkinson's disease.
Jakowec, M. W., P. Arevalo, M. Vuckovic, P. Turnquist, E. Hogg, J. Walsh[#], G. Akopian[#], C. Meshul*, A. Abernathy, M. Ramirez, B. Fisher and G. M. Petzinger.
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The adult brain possesses a tremendous capacity for activity-dependent neuroplasticity. Following injury to the brain, physical therapy plays an important role in promoting recovery. In neurodegenerative disorders such as Parkinson's disease, physical activity improves motor function and may lead to alterations in disease progression. To better understand the role of activity-dependent plasticity in brain repair we are investigating the application of intensive treadmill exercise training in the MPTP mouse model of basal ganglia injury and dopamine depletion. Mice were administered MPTP (4 20 mg/kg each) and subjected to intensive treadmill running for 30 days starting 4 days after the last injection of MPTP (when cell death is complete) at a speed up to 20 meters/minute for 1 hour. During the exercise paradigm, mice were investigated for improvement in behavioral motor features and learning. Harvested brain tissues were analyzed by HPLC for levels of dopamine and its metabolites, and glutamate and the pattern of expression of genes and proteins for tyrosine hydroxylase, dopamine transporter, dopamine receptors D1 and D2, and AMPA and NMDA glutamate receptors using western immunoblotting, immunohistochemistry, and in situ hybridization histochemistry. Electrophysiological analysis of dopamine release was determined using fast cyclic voltammetry on brain slices. Our findings indicated that there was an enhancement of both motor behavior recovery and rotarod learning in exercised mice despite no change in the number of SNpc dopaminergic neurons and the striatal levels of dopamine. Molecular analysis showed down-regulation of DAT and TH, and significant changes in the pattern of expression of ionotropic glutamate receptors in the cortex and striatum. In addition, exercise resulted in an increase in dopamine release compared to MPTP-lesioned mice without exercise. These findings demonstrate that intensive exercise can induce dramatic neuroplasticity in an animal model of basal ganglia injury and provides a valuable framework for supporting exercise in patients with Parkinson's disease.
Supported by grants to J. Walsh (RO1 AG21937), M. Jakowec (RO1 NS44327) and G. Petzinger (US Army NETRP W81XWH-04-1-0444).

(3) Society for Neuroscience Annual Meeting, Atlanta 2006 ABSTRACT #2

Changes in dopamine and glutamate electrophysiology in the MPTP-treated non-human primate and the exercised MPTP-treated mouse.
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We tested the hypotheses that dopamine (DA) and glutamate physiology are altered in the MPTP-treated squirrel monkey using electrophysiological methods. Fast cyclic voltammetry analysis of the monkey putamen revealed that MPTP treatment (6 weeks earlier) resulted in a dramatic loss in DA released in

response to intra-putamen stimulation (bipolar tungsten wire electrode, 0.1 msec 100-50 µA stimulus). Saline injected monkeys showed greater DA release in the lateral versus medial putamen. To determine if excitatory amino acid receptor-mediated physiology is altered in the MPTP-treated monkey putamen we applied whole cell voltage clamp techniques and examined the relative contribution of AMPA and NMDA receptors to corticostriatal synaptic events. Saline injected monkeys showed a relatively uniform NMDA/AMPA receptor ratio, while data from MPTP-treated monkeys suggested that two new populations emerged; one with a reduced NMDA/AMPA ratio and another with an enhanced NMDA/AMPA ratio. We applied a similar strategy to examine the impact of MPTP toxicity on DA and glutamate physiology in the mouse and, more importantly, to determine if changes striatal DA or glutamate physiology tracked the behavioral recovery induced by exercise in the MPTP-treated mouse. Cyclic voltammetry revealed a dramatic reduction in evoked DA release in the striatum of mice treated a month earlier with MPTP. A parallel group of mice were treated with MPTP and exercised daily on a treadmill. MPTP treated mice were significantly compromised in treadmill performance initially but achieved the same performance as saline injected mice by the end of one month of training. The exercise-mediated enhancement of motor skills transferred to a rotarod task. Prior work demonstrated exercise-induced suppression in striatal DAT immunocytochemistry in the MPTP-treated mouse (Fisher et al, 2004, J Neur Res 77:378), but voltammetry revealed a significant exercise-induced increase in DA release in the MPTP treated mouse. These data demonstrate emergent dopaminergic and glutamatergic plasticity created in the striatum following exposure to the neurotoxin MPTP. We hypothesize these forms of synaptic plasticity underlie both behavioral deficits created early as well as recovery seen later in the MPTP model.

This research is supported by grants to J Walsh (RO1 AG21937), M Jakowec (RO1 NS44327) and G Petzinger (US Army NETRP W81XWH-04-1-0444), and the Zumberge Foundation.

(4) Society for Neuroscience Annual Meeting, San Diego, CA, 2007. ABSTRACT #1

Dopamine treatment effects on neuroplasticity in the MPTP-lesioned Squirrel Monkey (*Saimiri sciureus*).

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The administration of the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to the Squirrel monkey (*Saimiri sciureus*) leads to the onset of parkinsonian symptoms due to the loss of nigrostriatal dopaminergic neurons and the depletion of striatal dopamine. Animals were administered a series of 3 s.c. injections of MPTP (2.0 mg/kg, free-base, 2 wks between injections) or saline as control. Starting 4 wks after the last injection of MPTP, when the time course of cell death is complete, saline and MPTP-lesioned animals were administered saline, levodopa plus carbidopa (7.5 mg/kg), or the dopamine agonist pramipexole (0.1 mg/kg) 5 days per wk for 4 wks followed by a 3 wks washout. A subset of animals underwent a series of microdialysis studies in conjunction with amphetamine challenge at pre-MPTP-lesioning, post-lesioning but before dopamine treatment, and 4 weeks after completion of dopamine therapy. During drug treatment all animals were subjected to a clinical rating scale to evaluate parkinsonian motor features. At the completion of the washout period brain tissues was collected from all animals and used for analysis of striatal dopamine levels using HPLC, perfusion fixed for immuno-EM, slice culture for electrophysiological studies, and proteins of interest including tyrosine hydroxylase (TH), dopamine transporter (DAT), vesicular monoamine transporter-2 (VMAT-2), and the effector molecule DARPP-32 using western immunoblot and immunohistochemical staining focusing on the caudate nucleus and putamen. Our findings show that MPTP-lesioned animals treated with Pramipexole or Sinemet showed (1) enhanced amphetamine evoked dopamine release; (2) normalization of Corticostriatal drive; (3) normalization of corticostriatal terminal glutamate density; (4) increased protein expression of TH, DAT, and VMAT-2 and (5) an increase in the phosphorylated forms of DARPP-32. Our data indicate that in addition to symptomatic treatment of parkinsonian motor features, both dopamine replacement therapy in the form of levodopa or dopamine agonists may lead to enhanced neuroplasticity in the MPTP-lesioned basal ganglia as indicated by the up-regulation of proteins important for dopamine biosynthesis, storage and transmission. The precise mechanism is currently unknown but may involve either direct

neurotrophic benefit via dopamine receptor stimulation or enhanced engagement of animals with their environment due to dopamine replacement therapy. This finding raises the issue that starting dopamine replacement therapy early in the course of Parkinson's disease may have additional benefit.
This research is supported by grants to G. M. Petzinger (US Army NETRP W81XWH-04-1-0444), J. Walsh (RO1 AG21937), and M.W. Jakowec (NIH RO1 NS44327-1).

(5) Society for Neuroscience Annual Meeting, San Diego, CA, 2007. ABSTRACT #2

The role of brain-derived neurotrophic factor (BDNF) over-expression in basal ganglia function and response to exercise in the MPTP-lesioned mouse model.

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Our previous work has shown that intensive treadmill exercise leads to improved motor performance in the MPTP-lesioned mouse model of basal ganglia injury. In addition, we have observed alterations in components of both the dopaminergic and glutamatergic neurotransmitter systems including altered patterns of expression of genes and proteins encoding receptor subunits as well as increased dopamine release with exercise in MPTP-lesioned mice subjected to intensive treadmill exercise. While the precise link between glutamate and dopamine neurotransmission with exercise is currently unknown, based on reports in the literature, we hypothesize that the neurotrophic factor BDNF may play a role. This factor is central to a number of important aspects of basal ganglia function including synaptogenesis, plasticity, and response to MPTP exposure. For these studies we utilized a transgenic mouse that over-expresses BDNF throughout the brain. BDNF-tg or C57BL/6 mice were administered MPTP in a series of 4 injections (20 mg/kg, i.p., 2 hours apart). A subset of mice was harvested 7 days post-lesioning for analysis of the degree of lesioning by examining protein expression of tyrosine hydroxylase and counting nigrostriatal dopaminergic neurons. Another subset of both BDNF-tg and C57 BL/6 mice were assigned to different groups including (1) saline injected, (2) saline + exercise, (3) MPTP injected, and (4) MPTP + exercise. The exercise regimen was initiated 5 days after the last injection of MPTP, when cell death is completed, and continued for 28 days (5 days/wk) achieving a rate of approximately 20 m/min for 60 minutes each session. Fast-scan cyclic voltammetry (FSCV) was used to examine electrically evoked dopamine release in striatal coronal brain slices. Using this method, dopamine release was sampled in five anatomically distinct sites that varied in dorsal to ventral and medial to lateral dimensions. Analysis of evoked dopamine release showed no release in non-Tg lesioned mice. However, BDNF-tg mice showed small amounts of dopamine release indicating a potential protective role by BDNF. We also examined long-term plasticity at corticostriatal synapses. Control mice demonstrated LTP in medial and LTD at lateral corticostriatal synapses. However, post-MPTP lesioning neither LTP nor LTD could not be evoked as has been reported for the 6-OHDA treated rat (Calabresi et al, 1992; Kreitzer and Malenka, 2007). Our data suggests BDNF over-expression accelerates the recovery of dopamine release and normal expression of long-term plasticity at corticostriatal synapses following exposure to MPTP.
This research is supported by grants to J. Walsh (RO1 AG21937), M.W. Jakowec (NIH RO1 NS44327-1) and G. M. Petzinger (US Army NETRP W81XWH-04-1-0444).

(6) Society for Neuroscience Annual Meeting, San Diego, CA, 2007 ABSTRACT #3

Altered AMPA receptor expression with exercise in the MPTP-lesioned mouse model of Parkinson's disease.

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We have previously demonstrated that intensive treadmill running leads to motor improvement in the 1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine (MPTP)-lesioned mouse model of Parkinson's Disease. In this study, we investigated changes in the pattern of expression of glutamate receptors and postsynaptic effector

molecules to elucidate the molecular modifications that influence the improvement seen in MPTP-lesioned mice after intensive exercise. Four groups of animals were used to examine these changes: (i) Saline; (ii) Saline + Ex; (iii) MPTP; (iv) MPTP + Ex. C57 BL/6 mice were administered four i.p. injections of MPTP (20mg/kg free-base, 2 hours apart) which yields 90% dopamine depletion in the striatum. Exercise was started 5 days (a time point when cell death is complete) after MPTP lesioning and continued for 28 days (5 days a week) using a motorized treadmill. At completion of the exercise regimen, tissue was harvested and the expression of mRNA transcript and protein for the α -amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) subtype of the glutamate receptor superfamily, as well as the effector molecule DARPP-32, was analyzed within the dorsolateral striatum. The changes in mRNA transcript expression of AMPA receptor subunits, including their alternative splice isoforms, flip and flop, were determined using qRT-PCR analysis. Using immunohistochemical staining, we examined the expression of AMPA receptor subunits, including their phosphorylated states, and the effector molecule DARPP-32. Our results indicate that exercise causes a downregulation of mRNA transcript in the pan forms of GluR1 and GluR2, and in the Flip isoform of the GluR2 subunit. Mice lesioned with MPTP also displayed decreased mRNA for GluR1 and GluR2. Immunostaining revealed changes not accounted for by transcript expression. No significant changes occurred in the expression of the GluR1 protein. We found an upregulation of the GluR2 protein subunit and its phosphorylated state (serine 880) in MPTP + Ex mice. The expression of the effector molecule DARPP-32, was downregulated in exercised mice. These studies showed that exercise influences the pattern of expression of the AMPA receptors within the striatum but that this phenomenon is not explained by mRNA transcript expression, suggesting that alternative mechanisms are involved in this process, such as protein interactions or localization in relation to the synapse. Findings from this study indicate that changes in AMPA receptor subunits may play a key role in putative molecular adaptations that are necessary for activity dependent synaptic plasticity in the dopamine depleted striatum, as is found in the Parkinsonian state.
This research is supported by grants to J. Walsh (RO1 AG21937), M.W. Jakowec (NIH RO1 NS44327-1) and G. M. Petzinger (US Army NETRP W81XWH-04-1-0444).

(7) Society for Neuroscience Annual Meeting, San Diego, CA, 2007 ABSTRACT #4

Memory impairment and affective behavior in the MPTP-lesioned mouse model of basal ganglia injury.
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Abstract: Depression, anxiety and dementia are common in patients with Parkinson's disease (PD). Molecular mechanisms connecting the loss of dopamine (DA) with mood and memory disorders are not well understood. The present study investigated whether the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced lesion of basal ganglia in mice can be used as an animal model of memory and affective dysfunction associated with PD. Established mouse behavior tests were used to compare control and MPTP-lesion mice. Depression was measured by tail suspension and sucrose preference. Anxiety was tested using light-dark preference and hole board, and fear was assessed with fear conditioning. Olfactory memory was tested by social transmission of food preference. Separate groups of adult male C57BL/6 mice were evaluated 7 and 30 days after MPTP lesion. Lesioning consisted of 4 i.p. injections of 20 mg/kg MPTP at 2 h intervals. This regimen has been shown to produce severe DA loss in the striatum (up to 90% loss) and 50-70% cell loss in the substantia nigra pars compacta. Control mice received 4 i.p. injections of saline. In the social transmission of food preference test, mice acquired information about novel flavor from a conspecific demonstrator. Subsequently, when presented with two unfamiliar flavors, control mice showed a strong preference (79.0 ± 3.3%) for the flavor consumed by the demonstrator. This preference was significantly decreased in mice 30 days after MPTP (58.7 ± 6.3%, $p < 0.05$), but not 7 days post-lesion (79.1 ± 3.3%). Fear conditioning at 7 and 30 days post-MPTP showed faster extinction of the freezing response to a tone compared to control mice. After 6 minutes of continuous tone exposure, control mice spent significantly more time freezing (43.8 ± 6.0%, $p<0.05$) compared to mice at 7 days (9.6 ± 3.2%) or 30 days post-MPTP (16.5 ± 7.3%). The tail suspension test showed a significant increase in percent of time spent in immobility 30 days compared to 7 days post-lesion (44.2 ± 3.2%, 29.3 ± 3.3%, $p<0.005$), but there was no difference between these two groups compared to control mice (36.6 ± 3.1%). There was no change in sucrose consumption between lesioned and control mice. There was no increase in time spent in the dark compartment in the light-dark preference test between

the groups. Similarly, there was no difference in the number of nose pokes in the hole board test between lesioned and control mice. Overall, these data suggest that the MPTP-lesioned mouse has potential to be used as an animal model of memory impairments associated with PD. On the other hand, acute treatment with MPTP does not induce significant changes in affective behavior in C57BL/6 mice.

(8) Society for Neuroscience Annual Meeting, Washington DC, 2008, ABSTRACT #1

Molecular mechanisms of glutamate neurotransmission after high intensity exercise in the MPTP mouse model of basal ganglia injury.

Michael W. Jakowec[1,2], Marta G. Vučković[3], Jon VanLeeuwen[1], Garnik K. Akopian[4], John P. Walsh[4], and Giselle M. Petzinger[1,2]

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Studies in our labs have shown that intensive physical exercise is beneficial for both patients with Parkinson's disease (PD) and animal models of basal ganglia injury (Fisher et al, 2004 and 2008; Petzinger et al 2007). However, the underlying molecular mechanisms are poorly understood. In this study we used the MPTP-lesioned mouse model of basal ganglia injury to investigate molecular mechanisms responsible for the beneficial effects of high intensity treadmill exercise. For this purpose, 8-10 week old male C57BL/6 mice were lesioned with 4 intraperitoneal (i.p.) injections of 20 mg/kg MPTP (free-base) or saline at 2 hr intervals. MPTP-lesioned and control mice were split into four experimental groups: (1) saline, (2) saline + exercise, (3) MPTP, and (4) MPTP + exercise. Treadmill exercise was initiated 5 days after MPTP-lesioning. Mice were habituated to run on a motorized mouse treadmill for 1hr daily and gradually trained to reach speed of 18m/min. Exercise was conducted 5 days per week for a total of 30 days of running. Electrophysiological studies showed exercise-induced reduction in excitatory post-synaptic currents of the medium spiny neurons in MPTP-lesioned mice, and decrease rectification in AMPA receptor conductance. Molecular analysis showed changes in the AMPA receptor subunit GluR2 and its phosphorylated state at Ser-880 supporting our electrophysiological findings. Using other techniques including qRT-PCR and western immunoblot analysis of striatal synaptoneurosomes (enriched for post-synaptic density complexes) we have documented changes in both mRNA transcripts and proteins important for striatal neurotransmission including PSD-95, synapsin I, and several glutamate receptor accessory proteins. Additionally, we have correlated these findings to changes in the morphology of medium spiny neurons in the dorsolateral striatum using the method of Golgi impregnation. Our hypothesis is that in MPTP-lesioned mice, high intensity treadmill exercise modulates the expression of glutamate receptors (especially the AMPA subtype) and normalizes glutamate neurotransmission in medium spiny neurons leading to decreased cortico-striatal hyper-excitability. These changes in glutamate receptors along with the changes we observe in the dopamine D2 receptor (see abstract from M. Vuckovic et al 2008) indicate that the experience-dependent neuroplasticity through intensive exercise has a dramatic influence on the injured basal ganglia and may represent a novel therapeutic target for modifying disease progression.

(9) Society for Neuroscience Annual Meeting, Washington DC, 2008, ABSTRACT #2

Neuroplasticity in the mptp-lesioned squirrel monkey (saimiri sciureus)

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Abstract: The purpose of this study was to examine molecular and neurophysiological correlates of striatal plasticity in the MPTP-lesioned nonhuman primate. In addition, we examined these same parameters in the context of dopamine replacement therapy using either L-dopa or dopamine agonist (pramipexole) administration. The administration of the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to the Squirrel monkey (*Saimiri sciureus*) leads to the onset of parkinsonian symptoms due to the loss of nigrostriatal dopaminergic neurons and the depletion of striatal dopamine. Animals were administered a series of 3 s.c. injections of MPTP (2.0 mg/kg, free-base, 2 wks between injections) or saline as control. Animals were harvested at either 2 or 12 months. Animals at 12 months demonstrated full behavioral recovery. In a subset of animals drug administration consisted of either levodopa plus carbidopa (7.5 mg/kg), or the dopamine agonist

pramipexole (0.1 mg/kg) 5 days per wk for 4 wks followed by a 3 wks washout. Microdialysis studies were conducted in a subset of treated animals to assess alterations in dopamine storage. Brain tissues analysis was carried out for striatal dopamine and glutamate levels using HPLC, slice culture for electrophysiological studies, and proteins of interest including tyrosine hydroxylase (TH), dopamine transporter (DAT), Dopamine Receptors D2 and D1, and AMPA-R and NMDA-R subunits using western immunoblot and immunohistochemical staining focusing on the striatum. Animals were examined for alterations in excitatory amino acid receptor-mediated physiology using whole cell voltage clamp techniques and examined for alterations in the input-output relationship between the intensity of stimulus delivered at the corpus callosum (input) and the size of the excitatory synaptic response (EPSC). Electrophysiological studies also examined the relative contribution of NMDA/AMPA receptor ratio. Our findings show that MPTP-lesioned animals either during behavioral recovery (12 month) or during drug treatment showed (1) normalization of Corticostriatal drive; (2) normalization of glutamate levels (4) alterations in dopamine storage and (5) alterations in AMPA and NMDA Receptor subunit expression. Glutamatergic and dopaminergic neuroplastic changes may be similar in both recovery and dopamine treated animals.

(10) Society for Neuroscience Annual Meeting, Washington DC, 2008, ABSTRACT #3

High Intensity Treadmill Exercise Normalizes Dopamine Neurotransmission in the MPTP Mouse Model of Basal Ganglia Injury.

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Physical exercise has beneficial effects on patients with Parkinson's disease (PD), but the underlying molecular mechanisms are poorly understood. Our previous work showed that high intensity treadmill exercise increases dopamine D2 receptor mRNA expression in the dorsal striatum of 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP) mouse model of basal ganglia injury. The present study used *in vivo* positron electron transmission (PET) imaging to investigate effects of high intensity treadmill running on dopamine receptor D2 function in MPTP-lesioned mouse. For this purpose, 8-10 week old male C57BL/6 mice were lesioned with 4 intraperitoneal (i.p.) injections of 20 mg/kg MPTP (free base) at 2 h intervals producing up to 90% dopamine loss in the striatum and 50-70% cell loss in the substantia nigra pars compacta. Control mice received 4 i.p. injections of saline. Lesioned and control mice were split into four experimental groups as follow: (1) saline, (2) saline + exercise, (3) MPTP, and (4) MPTP + exercise. Treadmill exercise was initiated 5 days after MPTP-lesioning. Mice were habituated to run on a motorized mouse treadmill for 1h daily and gradually trained to reach speed of 18m/min. Exercise was conducted 5 days per week for a total of 30 days of running. Three or four mice from each group were randomly selected for PET imaging with a high affinity D2 receptor ligand [^{18}F]-fallypride. Radioactive ligand was administrated to anesthetized mice via the tail vein. A 20 min transmission scan was collected immediately following ligand administration. Dynamic scans were collected over a time window of 90 min after the transmission scan. Our preliminary results show a significant increase in striatal D2 receptor binding potential (BP) in the MPTP-lesioned mice + high intensity treadmill exercise (BP = 7.8 ± 1.0) compared to MPTP-lesioned mice with no exercise (BP = 4.4 ± 0.2). Ongoing studies are investigating whether the increase in D2 binding potential in MPTP-lesioned mice is preserved for 6 weeks after the end of daily treadmill running. Overall, these data suggest that high intensity treadmill exercise has an effect in normalizing dopaminergic neurotransmission in injured basal ganglia. Findings from this study support a disease-modifying role of treadmill exercise and can be used to help design rehabilitation and physical therapy programs for patients with PD.

(11) Society for Neuroscience Annual Meeting, Washington DC, 2008, ABSTRACT #4

Affective and Motor Behavior in an 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Mouse Model of Basal Ganglia Injury

Lori M. Gorton, Marta G. Vucković, Nina V. Vertelkina, Giselle M. Petzinger, Michael W. Jakowec, Ruth I. Wood

Though largely classified as a progressive movement disorder, Parkinson's disease (PD) patients may exhibit a wide variety of non-motor, neuropsychiatric symptoms such as anxiety and depression. Exercise has been shown to improve psychological health in depressed populations but the mental health benefits of exercise in PD have not been thoroughly assessed. To investigate the relationship between dopaminergic nigrostriatal pathway degeneration and mood disorders, we lesioned C57Bl/6 mice with MPTP (4 X 20mg/kg free base, 2 h intervals) or gave saline vehicle (n=24/group). Mice were exercised 5 day/week for 6 weeks on a treadmill (8/group) for 1 hr. To control for the potential stress of forced exercise, an additional group of mice (n=8) was allowed to run voluntarily on a wheel for the same time period. Motor behavior was determined by daily total distances and average speeds, and overall rotarod performance after week 6. Depression and anxiety-like symptoms were assessed using established tests (sucrose preference, tail suspension, elevated plus maze, and marble-burying). **Results**: Wheel running mice ran significantly more than mice undergoing forced treadmill exercise, (30d avg =1.1 ± 0.05km vs. 0.44 ± 0.02km). MPTP lesion slightly reduced (1505 ± 97 vs. 1734 ± 41) while exercise increased overall Rotarod performance (ORP; 1660 ± 67 vs. 1481 ± 85.98). MPTP-lesioned mice buried more marbles vs. controls (62 ± 3.8% vs. 45 ± 6.6%); sedentary MPTP-lesioned mice buried the most marbles (74 ± 5.2%). The number of open and closed arm entries into the elevated plus maze was not different in any group. Neither MPTP lesion nor exercise had an effect on behavioral tests of depression. **Conclusion:** MPTP mice display subtle motor deficits 44 days post-lesion. MPTP mice may be more anxious than NaCl controls but show no indication of despair or anhedonia. Despite differences in the amount of running, neither forced nor voluntary exercise significantly improved measures of mood.

(12) Plasticity and Repair in Neurodegenerative Disorders, May 15-18, 2008, Lake Arrowhead, CA.

Molecular Mechanisms of Normalized Dopamine and Glutamate Neurotransmission After High Intensity Exercise in the MPTP Mouse Model of Basal Ganglia Injury.

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We are using the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of basal ganglia injury to investigate molecular mechanisms responsible for the beneficial effects of high intensity exercise on the dopaminergic and glutamatergic systems. We hypothesize that in MPTP-injured mice, high intensity treadmill exercise induces molecular adaptations in the basal ganglia, which normalize dopamine and glutamate neurotransmission. These changes may work synergistically to decrease cortico-striatal hyperexcitability in medium spiny neurons and underlie the beneficial effects of exercise. These studies support a disease-modifying role of exercise. Within the basal ganglia, cortico-striatal plasticity is proposed to be important for the control of motor function and for learning of motor skills. Previous studies suggested that dopamine receptor isoform D2, and glutamate receptors NMDA and AMPA could be key molecular candidates involved in cortico-striatal plasticity in medium spiny neurons of mouse striatum. Recent studies shown that high intensity daily exercise does not change the total DA levels in striatum, but increase stimulus-evoked DA release in striatal brain slices and D2 receptor mRNA in dorsolateral striatum. Positron Electron Transmission (PET) imaging will was used to quantify D2 receptor function *in vivo* at the level of the dorsal striatum in MPTP-lesioned and saline-treated mice after 30 days of high intensity treadmill exercise or no exercise. Three or four mice from each group were selected for imaging. For this study, a high affinity D2 receptor ligand [^{18}F]-fallypride was used. Ligand was administrated to anesthetized mice via the tail vein. Following a 30 min transmission scan, dynamic scans were collected over a time window of 90 min immediately following ligand administration. Our result show increased striatal D2 receptor binding potential in the MPTP mouse and is consistent with an increase in D2 protein expression. Exercise increased expression of GluR2 and phosphoGluR2-Serine880 in the MPTP mouse. Electrophysiological studies show exercise-induced reduction in excitatory post-synaptic currents of the medium spiny neurons in MPTP mice, and decrease rectification in AMPA receptor conductance. Findings support molecular analysis of GluR2 and an exercise induced decrease

in excitability of medium spiny neurons in the MPTP mouse. Ongoing studies are examining the expression of D2 receptor at the level of protein and mRNA in dorsolateral and ventral striatum using western blot, and rtPCR methods.

(13) Plasticity and Repair in Neurodegenerative Disorders, May 15-18, 2008, Lake Arrowhead, CA.

Voluntary and Forced Running Effects on Affective and Motor Behavior in an 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Mouse Model of Basal Ganglia Injury

Lori M. Gorton, Marta G. Vucković, Nina V. Vertelkina, Giselle M. Petzinger, Michael W. Jakowec.

Though largely classified as a progressive movement disorder, Parkinson's disease (PD) patients present with a wide variety of non-motor, neuropsychiatric symptoms such as anxiety and depression. Exercise has been shown to improve psychological health in depressed populations but the mental health benefits of exercise in patients with PD comorbidity have not been thoroughly assessed. To investigate the relationship between dopaminergic nigrostriatal pathway degeneration and mood disorders, we lesioned C57Bl/6 mice with MPTP (4 X 20mg/kg free base, 2 h intervals) or administered saline vehicle (n=24/group). One week later, mice were randomly assigned to a 5-day/week exercise regimen for six weeks, or to sedentary control groups. Exercised mice were run on a treadmill (8/group) with increases in time and distance so that a 6-week goal of two 30-minute sessions at a max speed of 18m/minute could be achieved To control for the potential stress imposed by forced exercise, an additional group of mice (n=8) was allowed to run voluntarily on a running wheel for the same time period. Motor behavior was compared by calculating daily total distances (expressed as average m/min) and overall rotarod performance after week 6. Depression and anxiety-like symptoms were assessed endophenotypically using established tests (sucrose preference, tail suspension, elevated plus maze with fecal boli quantitation, and marble-burying). **Results**: At the end of three weeks, exercised saline-treated animals (forced treadmill and voluntary wheel) showed an increased preference for 2% sucrose solution from baseline ($p<0.05$). No change in sucrose preference was observed in any of the MPTP-lesioned animals or in the saline sedentary group. Mice subject to forced treadmill exercise ran an average of 8 m/min for 50 minutes with an imposed max speed of 10m/min. By contrast, mice exercising voluntarily on running wheels ran significantly more (21.01 ± 1.3m/min for 50 minutes), and there were no differences between MPTP and control wheel-running groups. **Conclusion:** Preliminary results suggest that the motor deficits of MPTP-lesioned animals are not associated with decreased running distances. Also, After 3 weeks, we find no evidence of anhedonia in any group measured.

(13) Movement Disorders Society, Chicago, IL, June 2008.

Altered glutamate (AMPA) and dopamine (D2) receptor expression with treadmill exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse model of basal ganglia injury

G. Petzinger, M. Vukovic, A. Nacca, J. VanLeeuwen, G. Akopian, Q. Li, J. Walsh, R. Leahy, M. Jakowec Los Angeles, California

Objective: To determine the effects of high-intensity treadmill exercise (HITE) on striatal AMPA subunit (GluR1/GluR2) and D2 receptor expression using molecular analysis and PET imaging in the MPTP mouse model of Parkinson's Disease (PD).

Background: Exercise is beneficial for patients with PD. However, the underlying mechanisms and potential for disease modification are unknown. Alterations in dopaminergic and glutamatergic neurotransmission, induced by activity dependent (exercise) processes, may mitigate the cortically driven hyper-excitability in the basal ganglia normally observed in the parkinsonian state.

Methods: Mouse groups: saline; saline + exercise; MPTP; MPTP + exercise. Saline and MPTP mice were treadmill exercised (1hr/day; 28d). At exercise completion, dynamic PET scans were performed using ^{18}F-Fallypride (SA>6000Ci/mmole, 280uCi) and a microPET R4 scanner (90min) and images reconstructed using MAP algorithm. Binding potential (BP) was calculated using a dynamic model. Striatal tissue analysis included western blot analysis (D2) and qRT-PCR and immnuohistochemical staining (GluR1, GluR2, phosphorylated states). Whole cell voltage clamp methodology examined (i) input (stimulus strength)/output (excitatory post-synaptic current) relationship to evaluate synaptic strength (ii) rectification index to delineate AMPA-R subunit composition.

Results: HITE increased striatal D2 receptor BP in the MPTP mouse and is consistent with an increase in D2 protein expression. Exercise increased expression of GluR2 and phosphoGluR2-Serine880 in the MPTP mouse. Electrophysiological studies show exercise-induced reduction in EPSCs of MSNs in MPTP mice, and decrease rectification in AMPA receptor conductance. Findings support molecular analysis of GluR2 and an exercise induced decrease in excitability of medium spiny neurons (MSN) in the MPTP mouse.
Conclusions: HITE leads to alterations in dopaminergic and glutamatergic signaling within the injured basal ganglia. These changes may work synergistically to mitigate corticostriatal hyperexcitability in MSNs and underlie the anitparkinsonian effects of exercise. These studies support a disease-modifying role of exercise.

(14) Movement Disorders Society, Chicago, IL. June 2008

Sex differences in the MPTP mouse model of Parkinson's disease
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Objective: To investigate sex differences in anatomical and behavioral impairments in the MPTP mouse model of Parkinson 's disease.
Background: Parkinson 's Disease (PD) is a motor disorder resulting from the progressive loss of dopaminergic neurons of the substantia nigra pars compacta (SNC). Sex differences in PD have been reported in humans and in rodent models, with males being more impaired than females. Gonadal steroid hormones are thought, in part, to underlie this sex difference. The current study examined anatomical and behavioral sex differences in the MPTP mouse model of PD. We hypothesized that MPTP lesioning would produce more neuronal death in SNC of male mice, resulting in greater motor deficits relative to females.
Methods: Male and female mice were gonadectomized and received physiologic replacement with testosterone or estrogen to ensure constant hormone levels. Mice were injected with MPTP (10 mg/kg BW ip) or saline daily for 5 days. One week after the last injection, motor function was measured using the gait, pole, and rotarod tests. Immediately afterwards, animals were sacrificed. Caudal brain blocks containing SNC were immunostained for tyrosine hydroxylase (TH) and counterstained for Nissl.
Results: In unlesioned mice, males outperformed females on all three motor tests. Male mice had longer strides, descended the pole apparatus faster, and stayed on the rotarod longer than females. MPTP lesioning impaired overall rotarod performance (ORP) in both sexes. After MPTP treatment, ORP was equivalent in males and females (695 ±64 vs. 608 ±91, n.s.). Compared with unlesioned controls, MPTP-lesioned male mice had a more severe motor deficit than females (43% vs. 37%). MPTP treatment did not deplete TH neurons in SNC and there was no sex difference post-lesion.
Conclusions: MPTP lesioning produced a larger motor deficit in male mice than in females. These results support neurochemical studies in rodents showing more severe striatal dopamine depletion in males after MPTP. Furthermore, they support human gender studies that report a higher incidence and more severe PD phenotype in men. Interestingly motor dysfunction after MPTP was not accompanied by a parallel depletion of dopaminergic SNC neurons. This suggests that significant dopaminergic cell loss in SNC is not required to elicit behavioral deficits in motor performance.

(15) Society for Nuclear Medicine

Effects of intensive treadmill exercise on striatal D2 dopamine receptors binding in the MPTP-lesioned mouse model of Parkinson's disease
Nacca, A., Q. Li, M. Vuckovic, R. Leahy, P. Conti, B. Fisher, M. W. Jakowec, and G. M. Petzinger (2008) The. Society for Nuclear Medicine Annual Meeting, New Orleans.
Objective: To determine the effect of high intensity treadmill exercise on dopamine neurotransmission in the MPTP-lesioned mouse model of PD. Specifically the goal of this microPET study was to quantify striatal D2 receptors with the high-affinity radiotracer [^{18}F] Fallypride in male C57BL/6J mice undergoing high-intensity exercise versus no exercise.
Methods: For these studies there were four groups of mice (i) saline, (ii) saline+exercise, (iii) MPTP, and (iv) MPTP+exercise. Saline and MPTP-lesioned mice were subjected to motorized treadmill exercise (1 hour/day, 5 days/week for 6 weeks), starting 5 days after lesioning, when cell death is complete. We used the well-established D2 receptor PET ligand [^{18}F] Fallypride (S.A. 6000 – 10000 Ci/mmole and 270 -280 uCi per mouse) for imaging striatal D2 receptors at the end of the exercise period. Dynamic PET scans were performed with a microPET R4 scanner on anesthetized mice for 90 min after intravenous administration of the

tracer into the tail vein. Images were reconstructed using MAP algorithm with normalization and attenuation correction. Receptor quantification was preformed using the cerebellum as a reference region. The BP of [^{18}F] Fallypride was derived using the same dynamic model as [1] and [2].
Results: The [^{18}F] Fallypride binding potential of the striatal D2 DA receptor is reduced in the MPTP lesioned mouse (BP=4) compared to saline treated mouse (BP=10.5). Exercise leads to a normalization of the D2 DA receptor binding potential (BP=7) in the MPTP-lesioned mouse. These results are consistent with our molecular analysis showing an increase in the transcript and protein expression for the D2 DA receptor after exercise in the MPTP-lesioned mouse.
Conclusion: [^{18}F] Fallypride is a suitable probe to discriminate changes in the D2 DA receptor densities within the basal ganglia using the microPET R4 scanner. Exercise leads to changes in dopamine neurotransmission within the injured basal ganglia, as measured through alterations in binding potential using microPET imaging. Future studies will examine the effect of high-intensity treadmill exercise on D2 DA receptor density in individuals with PD using [^{18}F] Fallypride PET imaging.

(16) Society for Neuroscience, 2009, Chicago.

High intensity treadmill exercise regulate neurabin and spinophilin targeting of PP1 and AMPA subunit composition after dopamine depletion in the basal ganglia
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Protein Phosphatase 1 (PP1) plays an important role in the dopaminergic modulation of corticostriatal synaptic plasticity, including AMPA receptor subunit trafficking and spine morphology. Neurabin-1 and Spinophilin (Neurabin-II) are scaffolding F-actin binding proteins that target PP1 to the synaptic membrane and are highly expressed within medium spiny neurons (MSNs) of the dorsolateral striatum. Additionally, dopamine signaling facilitates F-actin stabilization and PP1 phosphatase activity by the interaction of dopamine D2 receptors (DA-D2Rs) third cytoplasmic domain with spinophilin. In the dorsolateral striatum, DA-D2Rs play an important role within the MSNs of the indirect projection pathway in modulating synaptic strength. Studies show that loss of dopamine correlates with loss of dendritic spines and failure to modulate a decrease in synaptic strength, including long term depression (LTD). AMPA subunits undergo changes in phosphorylation resulting in the insertion of GluR1 and the removal of GluR2. In the presence of dopamine, AMPA subunit composition induces changes in membrane permeability to glutamate hence a decrease in synaptic strength in MSNs. In a dopamine-depleted state, MSNs exhibit hyperexcitability due to a decrease in membrane permeability to glutamate and Ca^{2+}. It is believed that dopamine depletion leads to abnormal binding of spinophilin to PP1, which diminishes its phosphatase activity suggesting PP1 failure to dephosphorylate GluR1 at serine-845 and serine -831. However, the role that neurabin and spinophilin play in restoring normal synaptic strength due to experience-dependent neuroplasticity in a state of dopamine dysfunction is unknown. To investigate the hypothesis that neurabin and spinophilin are critical components of the mechanism required for exercise-dependent decrease in synaptic strength we designed shRNAmir vectors to knock down neurabin and spinophilin. These vectors were introduced in vivo to MSNs of the dorsolateral striatum via stereotaxic delivery. Chronic dopamine depletion was induced after a series of 4 injections of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) separated by 2 hours. High intensity exercise was initiated 5 days after MPTP-lesioning and consisted of 28 days of running on a motorized treadmill. Changes in expression of GluR1, GluR2, and their phosphorylated states were examined by western blotting and biotinylation assays. Electrophysiological recordings examined the amplitude and the ratio of AMPA/NMDA currents as a measure of synaptic strength in MSNs.

(17) Society for Neuroscience 2009 Chicago. IL.

PICK1-GluR2 Subunit Interactions in Experience-Dependent Neuroplasticity of the Basal Ganglia
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Dopamine dysfunction has been implicated in many diseases that involve dendritic spine loss and glutamatergic hyperexcitability, including Parkinson's disease, epilepsy, and autism. Alterations in AMPA receptor trafficking may underlie this dysfunction. PICK1 is a PSD protein located within glutamatergic synapses that is known to play a critical role in AMPA receptor trafficking through its binding and internalization of the phosphorylated glutamate receptor 2 serine 880 (GluR2-S880). Data from our lab has demonstrated that altered expression of GluR2 is accompanied by dendritic spine loss in medium spiny neurons of the mouse dorsolateral striatum in the context of dopamine depletion. Increased sEPSC amplitude is also observed in this state consistent with increased glutamatergic neurotransmission and changes in synaptic strength. Experience-dependent neuroplasticity in the form of intensive treadmill exercise reverses these findings. To understand the role of PICK1 in mediating the restoration of GluR2 in synaptic function and its potential role in the recovery of spine morphology, we are examining protein-protein interactions between these two constituents and other potential PSD regulatory proteins involved in spine morphology and synaptic strength. Chronic dopamine depletion was induced after a series of 4 injections of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) separated by 2 hours. High intensity exercise was initiated 5 days after MPTP-lesioning and consisted of 28 days of running on a motorized treadmill. The following groups (i) saline; (ii) saline plus exercise; (iii) MPTP; (iv) MPTP plus exercise were compared using co-immunoprecipitation, western immunoblotting, and immunocytochemistry.

(18) Society for Neuroscience, 2009, Chicago.

High intensity treadmill exercise modulates striatal medium spiny neurons activity in the indirect pathway of the mouse basal ganglia.
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Glutamatergic input from the motor cortex to the dorsal striatum is an important modulator of the basal ganglia activity. Medium spiny neurons (MSNs) in the dorsal striatum receive glutamatergic inputs from the motor cortex areas and dopaminergic input from the substantia nigra pars compacta. These two inputs converge onto the spines of MSNs and modulate their activity. Due to severe dopamine loss, patients with Parkinson's disease (PD) and animal models of basal ganglia injury develop aberrant striatal glutamatergic excitability, which is suggested to be a pathological maladaptation to neuronal injury. We hypothesize that behavioral activity such as regular daily exercise ameliorates cortico-striatal glutamatergic input into MSNs; however, the molecular mechanisms underlying this process are not well understood. Subunit composition of glutamate AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors and dopamine D2 receptor (D2-R), are critical for experience-dependent synaptic plasticity in the striatum. The present study focuses on their interaction in MSNs within the indirect pathway of the dorsal striatum, the part of the basal ganglia most severely affected by dopamine depletion. Our experimental approach utilized transgenic mouse line with eGFP expression selective to D2-R containing MSNs of the indirect pathway. Lesion to the basal ganglia was introduced by intraperitoneal injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a selective dopaminergic neurotoxin. Control mice (saline-injected) and lesioned mice (MPTP-injected) were trained to run daily on a mouse treadmill for 6 weeks, starting 5 days after the MPTP lesion. Four experimental groups included: (1) saline-injected; (2) MPTP-lesioned; (3) saline-injected + treadmill running; (4) MPTP-lesioned + treadmill running. Experience dependent synaptic plasticity was measured by utilizing the tools of electrophysiology, biocytin labeling, and protein expression assays. Our results indicate that high intensity treadmill exercise (a) alters spine density and dendritic arborization of MSNs predominantly within the indirect pathway of the striatum, (b) modulates AMPA receptor subunit composition in MSNs of the indirect striatal pathway, and (c) promotes AMPA GluR2 subunits insertion into the postsynaptic density of MSNs in the dorsal striatum. Studying exercise-induced modulations of the injured basal ganglia in animals will help develop a foundation for designing new intervention programs involving experience dependent neuroplasticity with the ultimate goal to modify progression of neurodegenerative disorders such as PD.

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(16) Petzinger, G. M., Fisher, B E., VanLeeuwen, J. E., Vuckovic, M., Akopian, G, Meshul, C. K., Holschneider, D. P., Nacca, A., Walsh, J. P., and M. W. Jakowec. (2009) Enhancing Neuroplasticity in the Basal Ganglia: The Role of Exercise in Parkinson's Disease. In Press, Movement Disorders.
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Manuscripts in Preparation:

(18) Vuckovic, M., C. Meshul, G. M. Petzinger, and M. W. Jakowec (2009) Restoration of dendritic spine density in striatal medium spiny neurons following exercise in the MPTP-lesioned mouse model.

(19) Vuckovic, M., Q. Li, A. Nacca, R. Leahy, B. Fisher, M. W. Jakowec and G. M. Petzinger (2009) High Intensity Treadmill Exercise Upregulates Striatal Dopamine D2 Receptor in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned Mice: *In vivo* PET Imaging Study. To be submitted to Journal of Neuroscience.

Selected Presentations:

(1) Petzinger, "Neuroplasticity in the MPTP-lesioned Nonhuman Primate", Workshop: Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, Feb19-22, 2004.

(2) Jakowec, " The Role of Exercise in Enhancing Neuroplasticity in the MPTP-lesioned mouse", Workshop: Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, Feb 19-22, 2004.

(3) Petzinger, "Enhancing Neuroplasticity in models of Basal Ganglia Injury", Van Der Muelen Symposium, University of Southern California, Keck School of Medicine, April 1, 2005.

(4) Petzinger, "Neuroplasticity and behavioral recovery in the MPTP-lesioned nonhuman primate" Workshop: Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, May 11-14, 2006.

(5) Petzinger, "Exercise-enhanced motor recovery in the MPTP-lesioned mouse model of Parkinson's disease". Parkinson Study Group Annual Meeting, Chicago, IL. October 2006.

(6) Petzinger, "Exercise, neuroplasticity, and animal models of Parkinson's disease". Parkinson Disease Foundation 50th Anniversary Research Symposium, New York City, October 2007.

(7) Jakowec, "Exercise and Parkinson's Disease", National Parkinson's Disease Research Symposium, San Diego, CA, November 2007.

(8) Jakowec, and Petzinger, Seminar and Workshop, Exercise in Parkinson's Disease, University of Pittsburg and The Parkinson's Disease Foundation. May 10-12, 2007

(9) Jakowec, Grand Rounds, University of Pittsburgh, Seminar, Neuroscience Program, University of Pittsburg, PA. Neuroplasticity in Parkinson's Disease: A Novel Therapeutic Target. December 5, 2007.

(10) Jakowec, Grand Rounds, Robert Wood Johnson Medical School, New Jersey. *Exercise induced Plasticity in Parkinson's Disease*. October 4, 2007

(11) Petzinger, Gait and Balance in Parkinson's disease. International Conference. Amsterdam. Exercise, plasticity and Parkinson's disease. February 3, 2008.

(12) Plasticity and Repair in Neurodegenerative Disorders May 15-18, 2008, Lake Arrowhead, CA
Symposium: Exercise, Plasticity, and parkinsonism.
Michael Jakowec, USC (moderator); Giselle Petzinger, USC; Diana Neely, Vanderbilt University; Daniel Holschneider, USC; Marjorie Ariano, Rosalind Franklin University.

(13) Winter Conference on Brain Research, Snowbird, UT January 26 to February 2, 2008
Session 80. Title: Exercise Enhanced Neuroplasticity in Parkinson's Disease and its Animal Models
Participants: Michael Jakowec, PhD (Chair) Univ. So. Cal; Giselle Petzinger, MD: Univ. So. Cal.; Charles Meshul, PhD: VA Medical Center/Oregon Health & Sciences University; Richard Smeyne, PhD: St. Jude, Memphis; Beth Fisher, PT/PhD: Univ. So. Cal

(14) Petzinger, PDF's 50th Anniversary Educational Symposium, Exercise and Parkinson's disease, New York, October 11-12, 2007.

(15) Petzinger, Parkinson's Disease Foundation, Taking Charge of Your Parkinson's, *Does Exercise Influence Parkinson's Disease?* April 4, 2009, Houston, Tx.

(16) Petzinger, Movement Disorders Society Annual Meeting, Paris France June 13, 2009
Presentation lecture: Exercise-enhanced plasticity in Parkinson's disease.

(17) Petzinger, XVIII WFN World Congress of Parkinson's Disease and Related Disorders, Miami FL. December 13-16, 2009. Session Chair on "Exercise and Parkinson's Disease".

Grant Application Submitted to the National Institutes of Health based on findings from this grant.

Active
2RO1 NS044327-05A1 (NIH) 6/15/09-6/14/11
PI: M. W. Jakowec (Co-PI G. M. Petzinger)
Glutamate-Dopamine Plasticity in Nigrostriatal Injury: Exercise Enhanced Recovery
Major Goal: The primary goal of this research proposal is to elucidate the molecular mechanisms underlying the interactions between dopaminergic and glutamatergic neurotransmission and the role that intensive exercise plays in mediating recovery following injury to the nigrostriatal dopaminergic neurons by the neurotoxicant MPTP.

Pending Grant Applications

1RO1 National Institutes of Health, NINDS
Central and Behavioral effects of exercise in patients with Parkinson's disease.
PI: G. M. Petzinger and B. Fisher
Submitted July 6, 2009
Project Summary/Abstract

While a number of studies have shown that exercise is beneficial in improving motor function in Parkinson's disease (PD), the central mechanism(s) by which this happens is poorly understood. Parkinson's disease is a problem of dopamine neurotransmission that results in corticostriatal glutamatergic hyperexcitability. There is compelling evidence that this hyperexcitable state underlies the very motor dysfunction associated with Parkinson's disease including gait and balance impairments, slowness and stiffness. Acting principally through the D2 receptor it is thought that dopamine can influence corticostriatal hyperexcitability by (i) reducing glutamate release pre-synaptically and (ii) diminishing the responsiveness of the medium spiny neurons within the striatum to glutamate, post-synaptically. Treatments that can restore dopaminergic signaling at D2 receptors as well as facilitate downstream molecular mechanisms can attenuate the hyperexcitable state and lead to improved motor function. Our preliminary data using intensive treadmill exercise in both animal models of PD and humans suggests that **exercise** facilitates dopaminergic signaling of the dopamine D2 receptor, decreases cortical excitability and improves motor function. **In this FIRST AWARD proposal for both principal investigators we will test the hypothesis that the benefits of exercise on motor performance are due to improvement of dopamine signaling and concomitant changes in cortical excitability**. This proposal will answer the following critical questions: (i) Does exercise have a central effect in early, untreated patients with PD; (ii) Is high-intensity exercise required for this central effect; (iii) is this effect related to behavioral improvement and (iv) is this effect specific to a dopamine-depleted state (Parkinson's disease)? The results of this study could have an immediate and substantial impact on the management of individuals with PD. This study supports the possibility that intensive exercise may delay the need for dopamine replacement therapy given the fact that the signal of dopamine may be magnified by the increase of the dopamine D2

For this proposal, we will randomize subjects with early stage untreated Parkinson's disease to one of three groups (n = 20 subjects per group): (1) High-intensity BWSTT (fast walking to jogging), (2) Low-Intensity BWSTT; (self-selected over ground walking speed) and (3) No exercise standard of care group. Subjects in the two exercise groups will exercise, one hour, three times per week for eight weeks (24 sessions). In order to determine the specificity of the central effects of exercise, 20 healthy subjects will undergo high-intensity BWSTT. Subjects will be examined at baseline and one week following exercise termination.

Specific Aim 1: to determine whether intensive exercise can produce changes in striatal dopaminergic neurotransmission in early untreated patients with PD.
Hypothesis 1A: High-intensity BWSTT leads to a greater increase in D2 receptor binding potential of ^{18}F-Fallypride in the putamen of subjects with PD compared with Low-intensity BWSTT and no exercise, standard of care.
Hypothesis 1B: High-intensity BWSTT leads to a greater increase in D2 receptor binding potential of ^{18}F-

Fallypride in the putamen of subjects with PD compared with healthy age-matched individuals.
Specific Aim 2: **to determine whether intensive exercise can produce changes in corticomotor excitability in early untreated patients with PD.**
Hypothesis 2A: High-intensity BWSTT leads to a greater increase in cortical silent period (CSP) duration using transcranial magnetic stimulation (TMS) in subjects with PD compared with Low-intensity BWSTT exercise and no exercise, standard of care.
Hypothesis 2B: High-intensity BWSTT leads to a greater increase in cortical silent period (CSP) duration using transcranial magnetic stimulation (TMS) in subjects with PD compared with healthy age-matched individuals.
Specific Aim 3: to determine whether intensive exercise can produce changes in motor performance in early untreated patients with PD.
Hypothesis 3A: High-intensity BWSTT leads to a greater change in walking performance and dynamic postural control during a complex movement (turning) in PD compared with Low-intensity BWSTT exercise and no exercise, standard of care.
Hypothesis 3B: High-intensity BWSTT leads to a greater change in walking performance and dynamic postural control during a complex movement (turning) in PD compared with healthy age-matched individuals.
Hypothesis 3C: High-intensity BWSTT leads to a greater decrease in either the total or the motor subscale of the Unified Parkinson's Disease Rating scale (UPDRS) score compared with Low-intensity BWSTT exercise and no exercise, standard of care.

The results from this award will have real impact on the management of Parkinson's disease given that findings from these studies will be essential for determining whether intense exercise leads to changes in dopamine signaling though increased D2 receptor expression that is accompanied by behavioral improvement. Since D2 activation is a critical and necessary component of motor function, findings from this study would emphasize that exercise should be regularly prescribed in Parkinson's disease in order to maximize dopamine signaling. This study will also reveal whether patients need to intensively exercise or whether low intensity exercise such as walking at a self-selected pace is sufficient to gain these central effects. Finally this study supports the possibility that intensive exercise may delay the need for dopamine replacement therapy given the fact that the signal of dopamine may be magnified by the increase of the dopamine receptor itself (i.e., dopamine D2 receptor) and therefore would be a critical first step in revealing the potential for exercise in modifying disease.

1RO1 HD060630-01
Functional Adaptation of Neural Circuits After Exercise and Basal Ganglia Injury
PI Holschneider (Co-PI Jakowec)
Submitted: March 2009 Score 2.5 (13th percentile) Pending Council review
Evidence suggests that the type of exercise and the way it is performed results in the recruitment of different motor circuits in the brain. Furthermore, in the presence of brain lesions, the ability of exercise training (ET) to recruit damaged circuits or to recruit alternate motor circuits and reorganize the sensorimotor map is modified. A systematic investigation on the relationship between ET and dynamic brain activation is lacking. The current proposal focuses on the compensatory cerebral responses elicited by ET after basal ganglia injury in a rat model. Key questions addressed are: (1) Do any of the histological and biochemical changes in the brain that have been linked to exercise, actually change neural function, and what are the protective effect of ET following basal ganglia injury? (2) Does ET elicit neural sprouting and angiogenesis? Where in the brain is this occurring? And how do specific parameters of the ET regiment (complexity, intensity, duration, forced or voluntary engagement) modulate the response? In addition, we will look at effects of cessation of ET and the efficacy of intermittent, low level ET in maintaining gains achieved during intensive training. Functional brain mapping during a locomotor challenge is used to examine the role exercise plays on the basal ganglia-thalamic-cortical (BGTC) and the cerebellar-thalamic-cortical (CbTC) paths, as well as on accessory sensorimotor areas. A novel, implantable, minipump developed by our team is used for timed injection of the CBF tracer [14C]-iodoantipyrine by remote activation in the freely moving animal. Regional CBF-related tissue radioactivity is quantified by autoradiography and analyzed in the three-dimensionally reconstructed brain. Motor skill assessment will track neurologic recovery. Tyrosine hydroxylase immunohistochemistry and cell counts will provide a measure of lesion extent, while measurement of GAP-43 will provide an assessment of exercise-related neural sprouting and synaptic plasticity. Regional measurements of vascular endothelial growth factor and vascular density will allow us to examine the role played by angiogenesis in response to ET.

At the end of the project, we will know to what extent specific parameters of ET determine regional changes in brain function, and what the impact is of basal ganglia injury on such functional changes. In addition, we will know if ET simply recruits existent cerebral circuits and vascular beds or if in specific brain regions, increased neural activation and/or neural sprouting elicits changes in the vascular anatomy? Together, these studies have wide-ranging impact for neurorehabilitation, neuroscience, and activity-based strategies aimed at augmenting the effects of ET.
PUBLIC HEALTH RELEVANCE: Exercise is helpful in improving the motor deficits after brain injury, however, little is known to what extent these effects are active at the level of the brain. This project uses an animal model of brain injury to address this gap in neurorehabilitation research. Specifically, it will examine what neural circuits of the brain are affected by exercise, whether its actions are mediated by direct effects on the nerves or through proliferation of blood vessels that carry nutrients to the areas of damage, what parameters constitute 'effective' exercise, and what is the persistence of any changes upon discontinuing exercise.

R21 NS067544-01
Synaptic strength and dendritic spine morphology in experience-dependent plasticity
PI Petzinger, Jakowec, Walsh, and Wang
Submitted July 30, 2009
Abstract: AMPA receptor trafficking plays an important role in modulating synaptic strength, spine density and morphology and may be affected by experience. The purpose of this R21 application is to investigate the role of two F-actin binding proteins, neurabin and spinophilin, in experience-dependent neuroplasticity of the striatum. Specifically spinophilin and neurabin interacts with the dopamine D2 receptor and (i) can regulate trafficking of AMPA-R subunits through binding and synaptic targeting of protein phosphatase 1 and (ii) spine density and morphology, through the regulation of F-actin polymerization. For this application we will examine the role of spinophilin and neurabin in experience dependent plasticity by investigating the effects of siRNA knockdown of spinophilin or neurabin in a novel transgenic mouse strain BAC-D2-EGFP marking the D2 indirect pathway of the basal ganglia subjected to dopamine depletion (by MPTP-lesioning) following intensive treadmill exercise. Dopamine depletion through MPTP administration leads to aberrant increase in synaptic strength, dendritic spine loss, and impaired motor function, due to impaired PP1 targeting and function. Using electrophysiological and immunohistochemical techniques our preliminary work demonstrates that experience through intensive exercise leads to (i) improved motor function (ii) decreased synaptic strength and (iii) restoration of dendritic spine density in the MPTP-lesioned mouse. This application will test the role of neurabin and spinophilin proteins as key components of the underlying mechanism. These studies are important since dopamine dysfunction contributes to many neurological disorders, including schizophrenia, autism, mental retardation, compulsive disorders, and Parkinson's disease and the etiology of these disorders may be influenced by experience-dependent neuroplasticity and in fact may represent a novel therapeutic target.
PUBLIC HEALTH RELEVANCE**:** Synaptic strength and spine morphology are influenced by experience-dependent neuroplasticity including those we observe with intensive treadmill exercise in our animal model of dopamine depletion. This work is important because understanding dysfunction in synaptic strength and spine morphology may underlie the etiology of many severe neurological disorders including schizophrenia, autism, mental retardation, and neurodegenerative disorders including Alzheimer's disease and Parkinson's disease.

1R21 NS066327-01 NIH NINDS
Defining the role of microglia in exercise-induced neuroplasticity following basal ganglia injury.
PI Lund (Co-PI Jakowec)
Abstract: Inflammation has long been recognized as a component of the histopathology and pathogenesis of Parkinson's disease and its animal models. However little is known about the role of inflammation, especially after the peak period of neuron death, when mechanisms of repair and plasticity are engaged. Activity-dependent intervention through high-intensity treadmill running following injury to the nigrostriatal region is associated with significant improvement in behavioral recovery and motor performance. The mechanism(s) involved in this benefit are not clear; it is not known what cells or molecules are involved, nor how exercise can enhance this behavioral recovery. In this application, we hypothesize that microglia activation is required for the beneficial effects of an intensive exercise regimen. We propose that activation of local microglia causes a change in the secretion pattern of cytokines, chemokines and neurotrophic factors thereby promoting

neuroplasticity and neural repair. The experimental approach consists of two specific aims. In aim 1 we will assess the effect of exercise on microglia activation during clinical recovery from basal ganglia injury. This will be accomplished by ex vivo analysis of microglia morphology and phenotype by fluorescence activated cell sorting (FACS), immunohistochemical confirmation of microglial proliferation and measurement of expression of key cytokines, chemokines and growth factors; correlations with measures of motor function recovery will be assessed. The second specific aim will determine the absolute requirement of microglia activation for exercise-enhanced recovery of motor function. We will assess recovery of motor function in mice receiving a treatment regimen that blocks microglia activation. PUBLIC HEALTH RELEVANCE Parkinson's disease is a severely debilitating disease for which there is no known cure. This study uses a mouse model of Parkinson's disease to examine the ability of exercise to promote recovery from Parkinson's disease by activating specific cells in the brain, called microglia. Findings from this study may help us to design future treatments for not only Parkinson's disease, but also disorders of the nervous system.

The Los Angeles Basin Clinical and Translational Science Institute (CTSI)
Pilot and Feasibility Grant Program: 2008-2009
PI: Petzinger
Support $50,000
The purpose of this proposal is to address gaps in our knowledge regarding the effects of exercise on motor behavior and to test the hypothesis that (i) high intensity treadmill exercise leads to a sustained increase in dopaminergic signaling, even when exercise is discontinued, and that (ii) high intensity exercise also leads to an increase in synaptic connections (increased dendritic spine density) in medium spiny neurons of the injured basal ganglia. We will validate intensity as an important parameter by comparing high to low.

These studies are important because they help identify important parameters of exercise that will be used for exercise and rehab studies in patients with PD in order to promote optimal basal ganglia function and motor recovery. Findings from this proposal will be directly translated into a NIH/ RO1 exercise study in subjects with PD, testing the hypothesis that intensity of exercise leads to increase in D2 expression, as measured through an increase binding affinity of 18-F Fallypride using PET imaging.
For this proposal we will utilize 6 groups of mice including (i) saline injected, (ii) saline + high intensity exercise, (iii) saline + low intensity exercise; (iv) MPTP-lesioned, and (v) MPTP-lesioned + high intensity exercise (vi) MPTP-lesioned + low intensity exercise. The exercise regimen consists high and low intensive running on a motorized treadmill starting 5 days after the last injection of MPTP, a time point when neurotoxin-induced cell death is completed. Mice will exercise 1 hour per day for 30 days (5 days per week). High-intensity exercise will consist of running for 1 hour at 25 m/min, and Low intensity exercise will consist of running for 1 hour at 5 m/min.

Specific Aim 1 will test the hypothesis that there is increase in striatal D2 receptor binding in MPTP-lesioned mice undergoing intensive treadmill exercise, and that this increase is sustained well after exercise is terminated. A subset of mice from all 6 groups will undergo PET imaging with 18F-fallypride at completion of a 30-day exercise regimen, and again after an additional 30 days of no exercise. High and low intensity exercise animals will be compared.

ORIGINAL ARTICLE

The Effect of Exercise Training in Improving Motor Performance and Corticomotor Excitability in People With Early Parkinson's Disease

Beth E. Fisher, PhD, Allan D. Wu, MD, George J. Salem, PhD, Jooeun Song, MS, Chien-Ho (Janice) Lin, PhD, Jeanine Yip, DPT, Steven Cen, PhD, James Gordon, EdD, Michael Jakowec, PhD, Giselle Petzinger, MD

ABSTRACT. Fisher BE, Wu AD, Salem GJ, Song J, Lin C-H, Yip J, Cen S, Gordon J, Jakowec M, Petzinger G. The effect of exercise training in improving motor performance and corticomotor excitability in people with early Parkinson's disease. Arch Phys Med Rehabil 2008;xx:xxx.

Objectives: To obtain preliminary data on the effects of high-intensity exercise on functional performance in people with Parkinson's disease (PD) relative to exercise at low and no intensity and to determine whether improved performance is accompanied by alterations in corticomotor excitability as measured through transcranial magnetic stimulation (TMS).

Design: Cohort (prospective), randomized controlled trial.

Setting: University-based clinical and research facilities.

Participants: Thirty people with PD, within 3 years of diagnosis with Hoehn and Yahr stage 1 or 2.

Interventions: Subjects were randomized to high-intensity exercise using body weight–supported treadmill training, low-intensity exercise, or a zero-intensity education group. Subjects in the 2 exercise groups completed 24 exercise sessions over 8 weeks. Subjects in the zero-intensity group completed 6 education classes over 8 weeks.

Main Outcome Measures: Unified Parkinson's Disease Rating Scales (UPDRS), biomechanic analysis of self-selected and fast walking and sit-to-stand tasks; corticomotor excitability was assessed with cortical silent period (CSP) durations in response to single-pulse TMS.

Results: A small improvement in total and motor UPDRS was observed in all groups. High-intensity group subjects showed postexercise increases in gait speed, step and stride length, and hip and ankle joint excursion during self-selected and fast gait and improved weight distribution during sit-to-stand tasks. Improvements in gait and sit-to-stand measures were not consistently observed in low- and zero-intensity groups. The high-intensity group showed lengthening in CSP.

Conclusions: The findings suggest the dose-dependent benefits of exercise and that high-intensity exercise can normalize corticomotor excitability in early PD.

Key Words: Basal ganglia; Central nervous system; Neuronal plasticity; Rehabilitation; Walking.

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BOTH BASIC RESEARCH and clinical studies suggest that high intensity (ie, high repetition, velocity, complexity) is a characteristic of exercise that may be important in promoting activity-dependent neuroplasticity of the injured brain, including the basal ganglia.[1,2] Activity-dependent neuroplasticity is defined as alterations within the CNS in response to physical activity that include such processes as neurogenesis, synaptogenesis, and molecular adaptations.[3] BWSTT is currently being studied as a treatment modality for promoting activity-dependent neuroplasticity and functional recovery in stroke and spinal cord injury in part because it can be used to manipulate intensity of practice.

In the last 7 years, there have been an increasing number of studies[4-10] that have examined the effect of treadmill exercise on PD, a debilitating and progressive neurodegenerative disease, characterized by motor slowness, stiffness, tremor, and balance dysfunction.[11,12] Improved motor performance has been reported and treadmill speeds have gradually increased from studies[4,6] in which subjects trained at self-selected velocities for comfort to speeds above overground walking velocity.

Although there has been a recent attempt to examine functional performance associated with higher training intensities,

List of Abbreviations

AAMHR	age-appropriate maximal heart rate
BDNF	brain-derived neurotrophic factor
BWS	body-weight support
BWSTT	body weight–supported treadmill training
CNS	central nervous system
CSP	cortical silent period
FDI	first dorsal interosseous
GABA	γ-aminobutyric acid
MEP	motor evoked potential
METS	metabolic equivalents
MMSE	Mini-Mental State Examination
PD	Parkinson's disease
PT	physical therapy
ROM	range of motion
TMS	transcranial magnetic stimulation
UPDRS	Unified Parkinson's Disease Rating Scale
USC	University of Southern California

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Presented in part to the Society for Neuroscience, October 17, 2006, Atlanta, GA; American Physical Therapy Association Combined Sections, February 1–5, 2006, San Diego, CA; the World Parkinson Congress, February 22–26, 2006, Washington, DC; and the National Parkinson Foundation Collaboration for Care Leadership Conference, October 20–22, 2005, San Francisco, CA.

Supported by the Kinetics Foundation and National Institute of Neurological Disorders and Stroke (grant no. K23-NS045764).

No commercial party having a direct financial interest in the results of the research supporting this article has or will confer a benefit on the author(s) or on any organization with which the author(s) is/are associated.

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0003-9993/08/xx0xx-00919$34.00/0
doi:10.1016/j.apmr.2008.01.013

few studies have used different levels of intensity to examine exercise-induced changes in functional performance and how they may compare.[7-10,13]

In addition, thus far exercise studies in PD have not examined for associated CNS changes. TMS is a noninvasive method of stimulating the brain and provides a tool with which to assess the excitability of the corticospinal motor system (corticomotor excitability). Single TMS pulses are applied over the motor cortex while recording surface electromyographic responses over a contralateral target muscle. If the target muscle is preactivated (contracted), the TMS pulse induces a characteristic transient period of electromyographic silence called the CSP. Silent period durations beyond 100ms are thought to reflect long-lasting cortical inhibitory processes. Important for this study, single-pulse TMS studies have shown systematic abnormalities of CSP and other corticomotor excitability measures in people with PD. In general, these abnormalities reflect higher corticomotor excitability in PD compared with controls.[14-17] Because CSP represents inhibitory influences on corticomotor excitability, higher excitability would be evident as a *shortened* CSP duration. In fact, shortened CSP durations are among the most consistent and widely reproduced TMS finding among PD patients.[18] Further, symptomatic treatment of PD with surgical or pharmacologic interventions is associated with lengthening of the CSP toward levels seen in control subjects.[19-21] These studies suggest that corticomotor excitability measures, particularly CSP durations, could underlie symptomatic improvement, such as improved motor performance. Thus, not only is TMS an excellent tool to measure CSP duration and to examine possible exercise-induced changes in PD, but more importantly TMS may be used to support the existence of CNS changes in response to different exercise parameters including intensity.

The objective of this feasibility study was to obtain preliminary data on the effects of high-intensity exercise on functional performance in people with PD relative to exercise at low and no intensity and to examine whether improved performance is accompanied by lengthening of CSP as measured through TMS.

METHODS

Participants

Thirty subjects with early-stage, Hoehn and Yahr stages 1 or 2 PD voluntarily consented to participate in the study.[22] Early stages of PD were chosen because (1) people with greater physical capability could engage in higher-intensity exercise and (2) neuroplastic mechanisms may be more robust and amenable to environmental influences.[23,24] All subjects were required to sign an informed consent document approved by the institutional review board at USC. Subjects were recruited largely from the USC Department of Neurology Parkinson's Disease and Movement Disorders Clinic and through newspaper and radio advertisements delivered to the greater Los Angeles area. Before enrollment the diagnosis of idiopathic PD was confirmed by a fellowship trained PD specialist.

The inclusion criteria for the study were the following: (1) diagnosis of PD within 3 years of study participation, (2) 18 years of age or older, (3) medical clearance from the primary care physician to participate in an exercise program, and (4) ability to walk. Potential participants were excluded if (1) the medical or physical screening examination showed a score of less than 24 on the MMSE,[25] (2) there were physician-determined major medical problems such as cardiac dysfunction that would interfere with participation, (3) they had musculoskeletal impairments or excessive pain in any joint that could limit participation in an exercise program, and (4) they had insufficient endurance and stamina to participate in exercise 3 times a week for a 1-hour session. For all participating subjects, all PD medications were kept stable during the course of the study.

Each subject was randomized into 1 of 3 groups: high-intensity exercise, low-intensity exercise, and zero-intensity (no-exercise) group. With their eyes closed, subjects were randomized by self-selecting a card corresponding to 1 of the 3 groups. Subjects were blinded to existing group assignments. Subjects in the 2 exercise groups received 24 sessions of exercise over 8 weeks by the same physical therapist. Heart rate and blood pressure were monitored during exercise to assess each subject's exercise tolerance and exercise intensity level. Level of intensity was defined in accordance with the Centers for Disease Control and Prevention and American College of Sports Medicine guidelines by MET, in which 1 MET is defined as the energy expenditure for sitting quietly.[26,27] In this study, low-intensity exercise was any activity that burned less than 3 METS and high-intensity exercise burned greater than 3 METS.

Procedures

High-intensity group. Subjects randomized to the high-intensity group participated in 24 sessions of BWSTT. Participants were fitted in a harness,[a] which was then connected to an overhead suspension system[b] positioned over a treadmill. The suspension system is an overhead-motorized pneumatic lift with a digital readout displaying the amount of weight support.[b] BWS was initially set at 10% of each participant's body weight (to take up the "slack" in the system). However, if the participant was unable to load and support his/her weight during stance with normal gait kinematics, the BWS was increased. Subjects were trained with the assistance of 1 physical therapist and 1 aide if necessary to assist with maintaining the trunk upright. The goal of each treatment session was to have the participant reach and maintain an MET level greater than 3.0 METS and/or 75% of an AAMHR using proper gait kinematics for stance and swing (upright posture, extending and flexing the hip, knee, and ankle and coordinating limb movements to achieve symmetric limb cadence and equal step length). The end goal (ie, at least by session 24) was that each subject would walk on the treadmill continuously for 45 minutes within the above MET level range. However, subjects were permitted to rest if necessary.

Progression on the BWS treadmill system occurred in a number of different ways. Within the limits of a person being able to walk with observationally normative gait kinematics, the following parameters were scaled up in difficulty: (1) BWS was decreased, (2) treadmill speed increased, (3) physical assistance was decreased, and (4) time on the treadmill was increased.

A physical therapist ran each treadmill session and was responsible for decisions regarding progression; monitoring upright posture, manual or verbal feedback on pelvic position, weight shift, stride characteristics, and cadence.

Low-intensity group. Subjects randomized to the low-intensity group participated in 24 sessions of PT. This group was representative of general or traditional PT for people with PD. An analysis of exercise studies in PD over a 50-year period showed that overall the physical demand of the exercise protocols for the most part were low to moderate in intensity. In addition, we were able to identify that the activities within the studies could be grouped into 6 categories: (1) passive ROM and stretching, (2) active ROM, (3) balance activities, (4) gait, (5) resistance training, and (6) practice of functional activities and transitional movements (ie, sit-to-stand).[28,29] Each 45-

minute session consisted of activities within each of these 6 categories. Activities were individualized for each subject based on specific impairments and subject goals and included but were not limited to (1) therapist stretch of hamstrings (passive ROM), (2) active stretch of calf in standing (active ROM), (3) standing on foam and single-limb standing exercises (balance), (4) overground gait training on linoleum floor and grass (gait), (5) rubber tubing exercises and weight lifting (resistance training), and (6) transfer training, sit-to-stand, and supine-to-sit (functional activities). MET levels for each activity were ascertained by either selecting activities that were listed in the Compendium of Physical Activities[30] or estimating MET levels for those activities that were not listed. For example, an active ROM activity might be a doorway stretch. The estimated METS for this activity would be 1.2, because it compares with standing quietly, a listed activity in the compendium. The goal of each treatment session was to have participants average 3.0 or fewer METS and/or heart rate of 50% or less of their AAMHR for 45 minutes, an MET level within the low-intensity exercise guidelines. Time spent in each activity was documented, and average MET level was calculated at the end of each session.

Zero-intensity group. The zero-intensity intervention consisted of six 1-hour education classes taken over an 8-week period. The following topics were presented: (1) Quality of Life: What is it? (2) Improving Quality of Life in Chronic Illness and PD; (3) Stress, Appraisal, and Coping; (4) Improving Memory; (5) Nonmotor Features of PD; and (6) Treatment Advances in PD.

Subjects in all groups were allowed to continue their customary exercise routines. They were asked, however, not to change their exercise routines. To monitor outside activity level, all subjects filled out a daily exercise diary that was reviewed by the treating therapist.

Data Collection

Data were collected before intervention and immediately after completion of exercise. Subjects began exercise or education classes within 1 to 2 weeks after the baseline assessment. All subjects took their customary medications at the same time relative to each assessment. All assessors were blinded to group assignment.

Assessments

Baseline clinical characteristics of the 3 groups of subjects were obtained and included age, duration of PD diagnosis, UPDRS score, MMSE score, and PD medications.

UPDRS and Hoehn and Yahr staging. Disease severity was examined preintervention and postintervention using the UPDRS and Hoehn and Yahr staging.[22,31] The UPDRS was completed by a second PD specialist, trained in performing the UPDRS. The side of the body (left vs right) and corresponding contralateral brain hemisphere that was most affected by PD was established using the UPDRS.

Functional assessments. All testing took place at the Musculoskeletal Biomechanics Research Laboratory at USC. All tests were performed by a blinded biomechanist. Biomechanic assessments of walking and sit-to-stand were used to better understand the underlying mechanisms by which any potential changes in functional capability occurred. Reflective markers (14-mm spheres) were firmly taped to the following bony landmarks: first toe, first and fifth metatarsal heads, medial and lateral malleoli, medial and lateral epicondyles of femur, greater trochanters, iliac crests, and L5-S1. In addition, noncolinear tracking markers were placed on the heel, lateral shank, and lateral thigh. An 8-camera (60-Hz) motion analysis system[c] recorded 3-dimensional coordinates of the pelvis, thigh, shank, and foot. Ground reaction forces were obtained from force platforms.[d] Three-dimensional marker-coordinate processing software (Workstation)[c] and Visual3D Movement Analysis Software[e] were used to process the raw coordinate data and compute the bilateral segmental kinematics and kinetics for the lower extremity.

Walking test. All participants were asked to walk at a *self-selected* pace and as *fast as possible* pace along the 10-m solid surface. For all participants, 3 trials were recorded and averaged for each condition. We computed average gait velocity (in m/s), step length (the distance between 2 successive heel contacts of the opposite feet, in meters), stride length (the distance between 2 successive heel contacts of the same foot, in meters), cadence (in number of steps/min), double-limb support time (in percentage of gait cycle), and ankle, knee, and hip sagittal plane maximum joint excursions (in degrees).

Sit-to-stand test. The sit-to-stand task was performed using a firm, armless, adjustable-height chair. The time from initial sitting position to the final sitting position at the end of 3 repetitions was recorded at a self-selected pace. All participants performed 3 sets. A total of 9 repetitions were recorded and averaged. For biomechanic analysis, ankle, knee, and hip sagittal plane extensor net joint moments (in Nm/kg) and joint power (in W/kg) were calculated. In addition, lower-limb symmetry was calculated as the absolute difference of right and left peak hip, knee, and ankle moments and power as well as ground reaction force during standing up.

Transcranial magnetic stimulation. Corticomotor excitability using TMS was assessed before and after the 8-week intervention. Additional criteria excluded those people in which TMS would be contraindicated such as presence of a pacemaker, metal in head, pregnancy, other neurologic disorders, current use of stimulants or medications known to lower seizure threshold, and personal or family history of seizure disorder.[32,33] All subjects participating in TMS signed a separate informed consent document approved by the institutional review board at USC.

Single-pulse TMS was applied using a figure-of-8 coil (9×5cm) with a Cadwell MES-10[f] by a blinded assessor certified as an experimenter in the TMS laboratory at USC. All TMS procedures were conducted on the more-affected side first. The TMS coil was held tangentially to the skull, with the handle pointing backward and laterally, perpendicular to the central sulcus.[34] Single pulses of TMS were delivered over the primary motor cortex while monitoring MEPs from the FDI muscle. The site that evoked the largest and most reliable MEP amplitudes was designated the motor hotspot. This location was marked on a Lycra cap fitted for each subject to ensure consistent targeting of this hotspot throughout the session. With the coil on the hotspot, stimulator intensity was systematically raised from 40% to 100% maximum stimulator output in 10% increments. At each intensity, 10 single TMS pulses were delivered every 5 to 10 seconds. Intensities were always delivered in ascending order. Electromyographic data were collected during isometric voluntary contraction of the FDI muscle at 10% of maximum voluntary contraction. Both subject and investigator visually monitored the level of muscle contraction, and the TMS pulse was timed to occur within 1 to 2 seconds of onset of the contraction while target level of contraction was maintained. Subjects were trained to maintain contraction after each TMS pulse until instructed to relax by the investigator. Electromyographic signals were acquired using surface electrodes in a belly-tendon montage from the FDI contralateral to TMS. The signal was amplified and band-pass

Table 1: Clinical Characteristics of PD Subjects at Baseline

Characteristics	Zero Intensity	Low Intensity	High Intensity
Patients (n)	10	10	10
Sex (male/female)	8/2	5/5	6/4
Age (y)	63.1±11.5	61.5±9.8	64.0±14.5
Hoehn and Yahr stage	1.9±0.3	1.9±0.3	1.9±0.5
Duration of PD (mo)	17.7±13.3	8.8±7.9	14.7±9.9
MMSE score	29.6±0.7	29.3±0.8	28.9±1.1
Medications			
Levodopa (mg)	90.0±202.5	15.0±47.4	115.0±226.1
Pramipexole (mg)	0.5±0.7	0.3±0.9	0.5±.1.0
Ropinerole (mg)	0	0	1.6±5.1
Amantadine	30.0±94.9	20.0±63.2	30.0±94.8
Selegiline	0.5±1.6	1.0±3.2	1.0±2.1

NOTE. Values are mean ± SD or as otherwise indicated.

filtered between 1 and 1000Hz. Data were stored for later analysis in 600-ms samples, beginning 100ms before TMS onset.[g]

TMS Data Analysis

All data were analyzed offline with a customized Matlab software tool[h] for analysis of time-series data (dataWizard).[35] Each TMS trial was analyzed for CSP duration. The CSP duration was defined as the time between the TMS pulse and the first return of rectified electromyographic activity of at least 50% of pre-TMS background activity after a period of sustained silence.[36] When no CSP could be discerned, CSP duration was marked as 0ms. For each subject and side, 10 CSP duration values per intensity were averaged. The relation between average CSP data and TMS intensity was fitted to a sigmoid curve.[37,38] Sigmoid curves were summarized by 3 parameters: maximal CSP duration (CSPmax), maximal slope (CSPslope), and a midpoint intensity where CSP duration is half maximum (CSP_{50}).

Statistical Analysis

Because this study was conducted as a preliminary trial to assess the responsiveness of people with early-stage PD to high-intensity exercise and observe for changes in measures of brain and behavior, only descriptive analyses including mean and SD were conducted. Percentage change (mean and SD) were calculated as the (post value – pre value)/pre value × 100 for each subject. Observed trends are reported for the individual exercise groups.

RESULTS

Treatment Groups

All 30 subjects completed the study with no adverse events. Over the 24 exercise sessions, the high-intensity exercise group worked on average at an MET level of 4.3 with a range between 2.5 and 13.3 METS. In fact, 60% of the high-intensity exercise subjects reached 8.0 to 13.3 METS while running at 0% grade and speeds ranging from 8.0 to 12.8km/h (5.0–8.0mph). Heart rate for 7 of the high-intensity subjects ranged between 70% and 75% of AAMHR within and across exercise sessions. For the remaining 3 subjects treated with β-blockers, heart rate ranged between 50% and 60% of AAMHR. The 10 subjects in the low-intensity exercise group averaged 2.4 METS over 24 sessions. The range of intensity of exercise was between 1.8 and 2.7 METS for the low-intensity exercise group. Heart rate for the low-intensity subjects was consistently at 50% or less of AAMHR across and within exercise sessions.

All subjects in the zero-intensity group attended all education classes. The first 4 subjects randomized to the education arm of the study attended education classes as a group. The remainder of the participants received the same education classes, but they were conducted for each subject.

Clinical Characteristics

The mean baseline clinical characteristics of the 3 groups of subjects are shown in table 1. It can be seen that the groups were similar at baseline in age, Hoehn and Yahr stages, duration of PD, and MMSE. Although there was variability within and between groups regarding PD medications, no PD medications were adjusted during the trial. The baseline and postexercise means and SD in UPDRS total score and UPDRS subscores are shown in table 2 for each exercise group. Both UPDRS total and motor scores were slightly lower for each of the 3 groups at the postexercise time point.

Motor Performance

All groups showed improvement in some gait performance measures (tables 3, 4). However, the high-intensity group showed consistent improvement in most gait parameters. Specifically, the high-intensity exercise group showed preexercise to postexercise increases in self-selected gait speed (4.4%), stride length (4.7%) (fig 1A), and step length (5.8%). Hip (fig 1B) and ankle (fig 1C) joint excursion increased by 7.5% and 4.6%, respectively, after 24 sessions of BWSTT. In addition, double-limb support time decreased by 6.3% (ie, increased single-limb support). Similar to the results of the self-selected speed condition, in the fast walking assessment the high-intensity exercise group showed within-group postintervention increases in stride length (4.8%), step length (5.6%), and hip (7.4%) and ankle (8.5%) joint ROM.

The average time to accomplish 3 repetitions of sit-to-stand decreased slightly for all groups. We were unable to detect any pre–post differences in lower-extremity symmetry (left vs right lower extremity) at the hip, knee, and ankle joints during sit-to-stand because of the high variability of these measures. However, although both the zero- and low-intensity groups showed decreased symmetry of ground reaction force (82.4% and 5.1%, respectively), the high-intensity group had a 33.3% increase in symmetry, suggesting more equal distribution of

Table 2: UPDRS

UPDRS	Zero Intensity	Low Intensity	High Intensity
UPDRS total			
Baseline	36.1±9.5	39.4±9.3	35.9±13.3
Postexercise	32.9±10.6	34.2±8.0	33.8±14.6
UPDRS mental			
Baseline	0.8±0.79	1.6±1.2	1.4±2.1
Postexercise	1.1±0.99	1.7±1.5	1.4±1.8
UPDRS ADLs			
Baseline	7.7±3.8	7.3±2.9	7.0±3.3
Postexercise	6.8±4.0	5.8±2.7	7.6±3.9
UPDRS motor			
Baseline	27.6±7.3	30.5±8.7	27.6±10.3
Postexercise	24.9±8.8	26.7±7.5	24.8±9.0

NOTE. Values are mean ± SD.
Abbreviation: ADLs, activities of daily living.

Table 3: Kinematic Variables During Walking

Outcome Measure	Zero Intensity	Low Intensity	High Intensity
Velocity (m/s)			
Baseline	1.39±0.17	1.40±0.18	1.46±0.20
Postexercise	1.41±0.17	1.42±0.17	1.52±0.19
Step length (m)			
Baseline	0.68±0.11	0.71±0.08	0.73±0.10
Postexercise	0.71±0.11	0.72±0.07	0.77±0.08
Stride length (m)			
Baseline	1.37±0.23	1.42±0.15	1.48±0.18
Postexercise	1.41±0.23	1.44±0.14	1.54±0.16
Step width (m)			
Baseline	0.12±0.02	0.10±0.02	0.11±0.02
Postexercise	0.11±0.02	0.10±0.02	0.11±0.02
Cadence (steps/min)			
Baseline	120.33±9.26	120.57±11.60	120.66±10.40
Postexercise	121.09±8.60	118.94±10.20	120.85±8.50
Double-limb support time (% of gait cycle)			
Baseline	24.04±6.17	19.53±4.49	21.20±3.35
Postexercise	21.22±4.03	19.87±3.58	19.68±2.58
Hip ROM (deg)			
Baseline	40.1±5.3	39.6±4.4	41.22±5.5
Postexercise	39.1±4.2	41.0±4.6	44.25±6.2
Knee ROM (deg)			
Baseline	64.0±4.1	63.4±5.1	66.0±5.8
Postexercise	64.6±4.2	64.5±4.5	64.8±4.8
Ankle ROM (deg)			
Baseline	24.2±5.2	27.6±2.5	29.0±3.4
Postexercise	24.4±5.5	28.6±3.4	30.3±3.3

NOTE. Values are mean ± SD.

body weight between the lower extremities during the sit-to-stand task.

Transcranial Magnetic Stimulation

A subset of subjects from each group participated in TMS testing (4 in zero-intensity, 7 in low-intensity, 5 in high-intensity). Both the more- and less-affected hemispheres were tested. No subject had any side effects from TMS. There was no differential effect of intensity of exercise on CSPslope or CSP_{50}. However, for CSPmax, the high-intensity group had an average increase in maximal CSP duration for the more-affected hemisphere (32ms) compared with a 17-ms decrease for the low-intensity group and no change for the no-exercise group (black lines in fig 2). Increases in CSP duration were seen in all subjects who had undergone high-intensity exercise (mean ± SD: pre, 175.4±35.3ms; post, 207.3±27.1ms). The same pattern for the high-intensity group was observed for the less-affected hemisphere with an average 19.4-ms increase in CSP after the exercise intervention. The zero-intensity group showed an average 6.5-ms decrease, and there was no change for the low-intensity group. There was 1 subject each in the zero- and low-intensity groups who showed pronounced shortening of CSPmax. On close inspection, the data from these 2 subjects were deemed valid, and the discernible shortening seen potentially reflects the variability between subjects who are within a 3-year range of PD diagnosis.

DISCUSSION

In this study, we found that people with PD participating in high-intensity BWSTT improve spatiotemporal gait parameters, kinematics of gait performance, and lower-extremity symmetry of ground reaction force in sit-to-stand task. This improvement was not consistently observed across measures in the other exercise intensity groups. In addition, every subject undergoing high-intensity BWSTT showed a lengthening of CSP. Lengthened CSP was not consistently observed in subjects in the zero- and low-intensity groups, suggesting that high-intensity exercise may induce activity-dependent neuroplasticity as measured through changes in corticomotor excitability. Finally, all subjects in the high-intensity group completed the exercise program, showing that subjects with PD can be challenged at and can tolerate a very high-intensity treadmill exercise program.

All subjects showed improvement in gait velocity during both self-selected and fast-paced walking, which was most evident in the high-intensity group. Similar to our results, some improvement in gait velocity during self-selected speed walking has been reported in other studies examining treadmill exercise effects in PD. The modest improvement in gait velocity *after* treadmill exercise reported in all these studies including ours may be related to the fact that subjects were already walking at normal gait velocities *before* the exercise programs.[39-42] Perhaps a more notable observation is that subjects in the high-intensity exercise group showed improved gait performance manifesting as changes in gait strategy. Thus, the high-intensity subjects walked within the range of normal gait speed but did so by taking longer steps and moving forward over their limbs through a larger ROM (see tables 3, 4). These changes in gait performance driven by high-intensity practice were not observed to the same degree in the low- and zero-

Table 4: Kinematic Variables During Fast Walking

Outcome Measure	Zero Intensity	Low Intensity	High Intensity
Velocity (m/s)			
Baseline	1.96±0.38	1.92±0.23	1.91±0.32
Postexercise	2.04±0.40	1.94±0.19	2.00±0.34
Step length (m)			
Baseline	0.81±0.17	0.82±0.11	0.84±0.11
Postexercise	0.82±0.13	0.85±0.09	0.88±0.11
Stride length (m)			
Baseline	1.60±0.31	1.65±0.22	1.66±0.21
Postexercise	1.64±0.26	1.65±0.12	1.74±0.24
Step width (m)			
Baseline	0.12±0.03	0.10±0.02	0.10±0.03
Postexercise	0.11±0.02	0.10±0.02	0.11±0.02
Cadence (steps/min)			
Baseline	147.45±18.47	141.37±13.91	138.65±11.50
Postexercise	151.11±20.23	142.12±14.26	138.71±9.87
Double-limb support time (% of gait cycle)			
Baseline	17.36±4.40	16.12±4.51	16.27±4.14
Postexercise	13.59±3.82	15.80±3.22	15.48±2.87
Hip ROM (deg)			
Baseline	45.0±5.3	45.2±5.9	47.2±6.5
Postexercise	44.6±4.9	46.7±5.0	50.4±6.3
Knee ROM (deg)			
Baseline	64.0±7.8	62.0±6.0	67.1±6.7
Postexercise	60.2±13.4	61.7±7.2	66.9±5.6
Ankle ROM (deg)			
Baseline	26.3±4.3	28.3±5.3	30.3±4.7
Postexercise	26.4±4.4	29.5±5.7	32.5±3.3

NOTE. Values are mean ± SD.

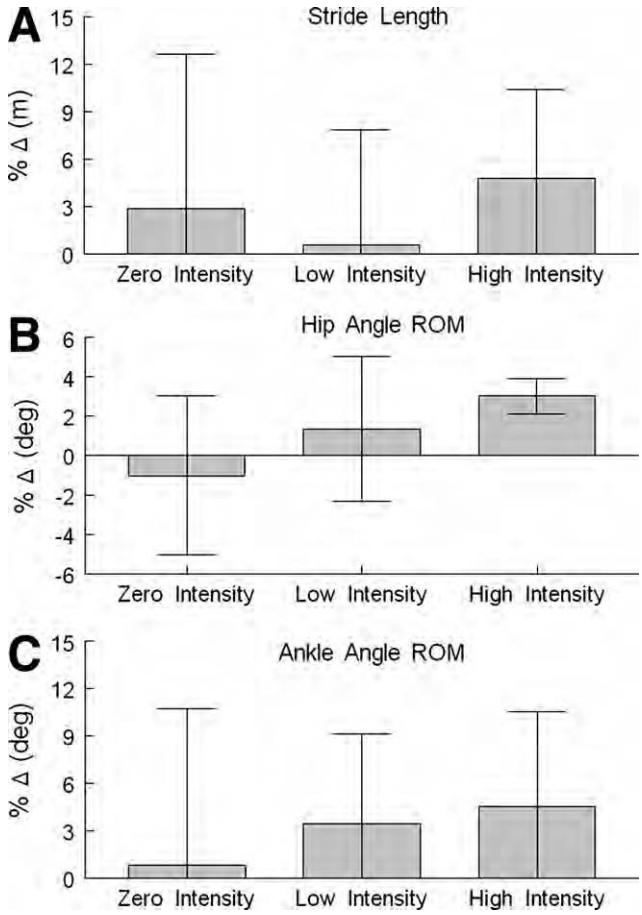

Fig 1. The percentage change (post- pre-exercise/preexercise × 100) in motor performance measures are shown for each of the 3 exercise groups: zero, low, and high intensity. (A) Percentage change in stride length (in meters) for each group. Stride length is the distance from right-heel contact to the following right-heel contact. (B) Percentage change in hip angle ROM (in degrees) or hip joint excursion for each group. (C) Percentage change in ankle angle ROM (in degrees) or ankle joint excursion for each group.

intensity groups and represent a pattern that is in notable contrast to the problematic gait pattern of people with PD (ie, short steps and limited advancement forward over the lower limbs). Similar findings were reported by Pohl et al,[9] in which relatively high-intensity treadmill training compared with conventional gait therapy was shown to have effects on gait pattern, specifically stride length and double-limb support time.

From our study, we learned that increased gait speed may not be a sufficient measure to determine the success of a treadmill intervention in early stage PD and that use of a wide range of gait parameters may be necessary to observe improvement in essential characteristics of gait. In addition to gait measures used in our study, measures of swing and stance time variability (coefficient of variation) have been shown to be particularly sensitive at detecting abnormalities in early PD.[43] Herman et al[8] recently observed that these measures of variability may be modified by an exercise intervention.

In addition, the high-intensity group showed increased symmetry of ground reaction forces in the sit-to-stand task. This suggests that high-intensity treadmill exercise may lead to improvement in other nongait motor tasks and that the treadmill training may assist in the ability to load the lower limbs equally. Although treadmill studies in PD[6,7,10,13] have examined other standing or upright axial tasks in addition to gait performance, to our knowledge this study is the first to report a benefit in another distinct motor task that may be due to intense gait training exercise.

In our study, there did not appear to be detectable postintervention differences in the UPDRS between the exercise groups, which was in contrast to the more consistent improvement in functional performance tests observed in the high-intensity group. Possible reasons for differences in outcome measures may be due to (1) the large degree of variability in the UPDRS among people within 3 years of diagnosis, along with the small sample size, and (2) greater sensitivity of functional and objective measures compared with the more subjective UPDRS in capturing changes in motor performance.

An important objective of this study was to examine if high-intensity exercise may be accompanied by CNS changes. Using TMS, we found lengthening of maximal CSP in all subjects in the high-intensity exercise group that was not consistently observed in subjects in the zero- and low-intensity groups. This finding suggests that intensity may be an important exercise parameter for facilitating activity-dependent neuroplasticity in association with improved motor performance.

Among TMS studies examining corticomotor excitability in PD patients, CSP durations are among the most consistent abnormalities reported, with generally shorter duration associated with greater parkinsonian symptoms.[44] CSP durations are usually shorter in PD patients compared with controls and, within PD patients, are shorter on the more-affected side compared with the less-affected side.[14,45] Both CSP durations and parkinsonian symptoms are sensitive to dopaminergic medication. Similar to the observed effects of high-intensity exercise, CSP durations are prolonged in PD patients after taking levodopa,[45-47] apomorphine,[48] and pergolide,[19,49] drugs known to provide effective symptomatic relief of motor symptoms. As clinical improvement accompanies dopaminergic treatment, this clinical improvement therefore corresponds with an increase in CSP duration.[45]

The level of excitability within the motor cortex is a balance between excitation and inhibition. The late part of the silent period duration is thought to reflect long-lasting cortical inhibitory processes.[50] As such, the duration of the CSP has been used as an index of the strength and time course of these processes. Studies have shown a loss of cortical excitation and inhibition balance in PD with higher motor system excitability in patients with PD at rest compared with controls—most likely the result of reduced intracortical inhibition.[14,15,44] Under tonic muscle contraction, shortened CSP in PD also suggests an enhanced excitability.[14] Inhibition as measured through CSP may be important for suppressing competing motor networks, thereby facilitating cortical-basal ganglia loops specified for an intended motor action.[44] Loss of inhibition as the result of loss of inhibitory cortical mechanisms (ie, shortened CSP), as seen in PD, may impair the focus of neuronal activity onto the appropriate pathways and enhance unspecific motor program transmission. Therefore, interventions such as intense exercise that lengthens CSP may help restore normal motor processing.

The mechanism underlying the lengthening of the silent period duration in people with PD undergoing high-intensity exercise is unclear. However, it is known that CSP is mainly mediated by GABA-B receptors.[44] GABA is the major inhibitory neurotransmitter in the basal ganglia, and abnormalities of GABAergic transmission are key aspects of the pathophysiology of movement disorders that involve the basal ganglia.[51] In

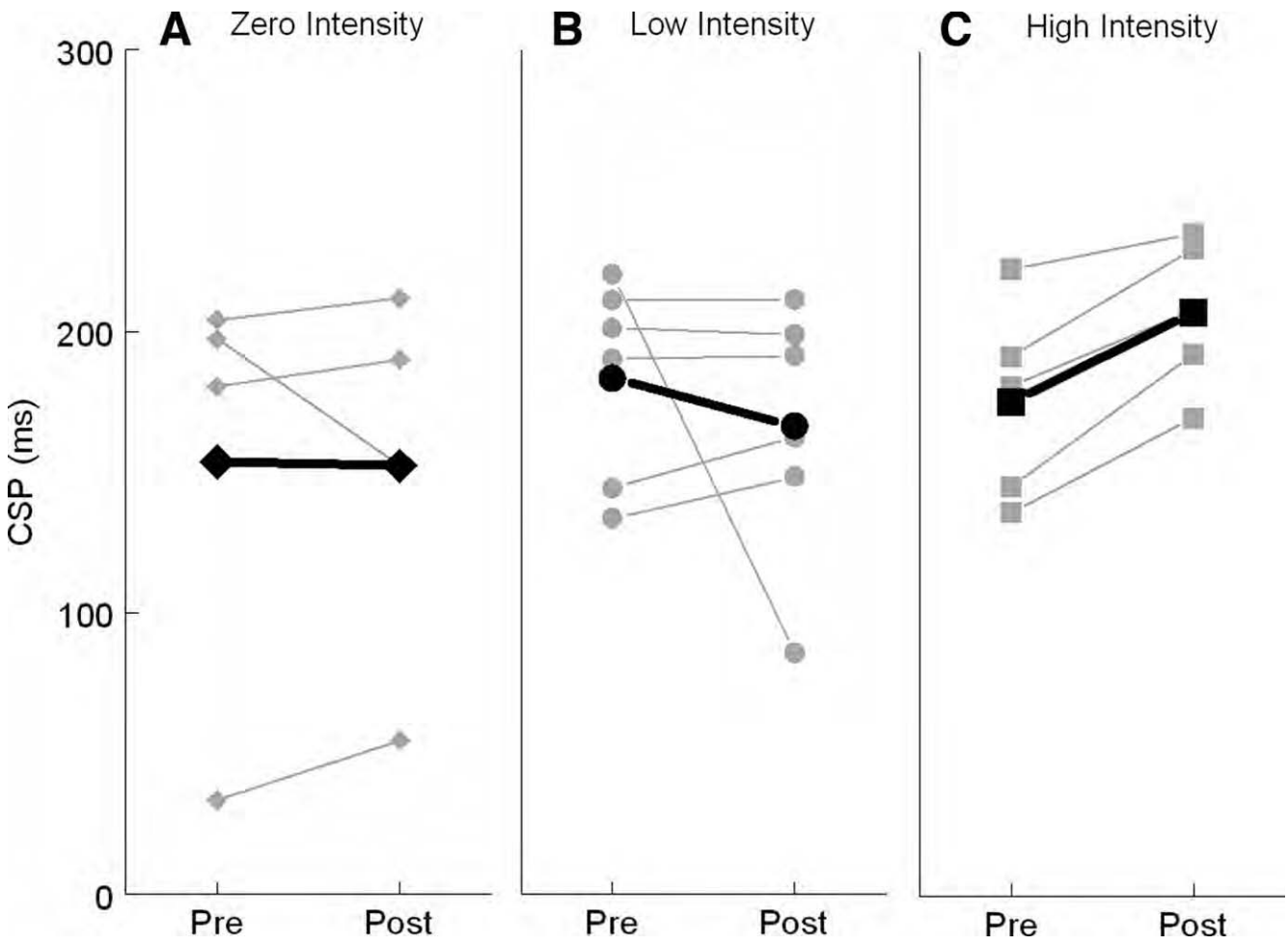

Fig 2. Pre- and postexercise measures of maximal CSP duration (in milliseconds) for subjects in the (A) zero-intensity, (B) low-intensity, and (C) high-intensity groups. Four subjects within the zero-intensity group participated in the TMS studies compared with 6 subjects in the low-intensity and 5 subjects in the high-intensity exercise groups. The thick black lines represent the average pre-CSP and post-CSP for each group.

addition, voluntary exercise can increase levels of BDNF. BDNF has neurotrophic and neuroprotective properties, can enhance brain plasticity, and appears to be a prime candidate for mediating the long-term benefits of exercise on the brain.[52,53] BDNF enhances neuronal function by promoting synaptogenesis and neurogenesis.[54] Evidence has shown that BDNF modulates the level of functional inhibition in an activity-dependent manner by regulating the number of GABAergic interneurons.[55] Although the role of BDNF in modulating GABA-mediated inhibitory transmission is not fully understood, it is possible that the lengthening of CSP in this study is related to an exercise-induced increase in BDNF.[56]

In our study, we found that the increase in CSP duration for the high-intensity group was observed in an intrinsic hand muscle, whereas the intense exercise involved a lower-extremity task, specifically treadmill training. This finding suggests that CSP may serve as a marker for a more generalized change in the CNS as a function of a primarily lower-extremity intervention. This is not the first report of postintervention and, specifically, post–treadmill training effects on corticomotor excitability using TMS in people with neuropathology. Changes in corticomotor excitability and associated improvements in walking function were found after intensive treadmill training both in people with spinal cord injury[57,58] and stroke.[59] However, to our knowledge, this is the first demonstration of exercise-induced changes in corticomotor excitability using TMS in people with PD, a progressive neurologic disorder. Animal models of PD have also supported activity-dependent neuroplasticity after intensive treadmill training as measured through changes in dopamine handling and neurotransmission, including increased dopamine release, decreased uptake, and an increase in the postsynaptic dopamine D2-receptor subtype within the basal ganglia.[2,60]

Study Limitations

An important limitation of this study is the small sample size and large variability in disease severity and baseline motor performance. As a result of this variability we were not able to show group differences using inferential statistics. Nevertheless, we were able to show benefits of high-intensity exercise in motor performance and corticomotor excitability. This trend of changes we observed warrants further study with a larger sample size to allow for a more discerning statistical analysis and determination of the relationship between changes in corticomotor excitability and motor performance.

CONCLUSIONS

The interest to promote neuroplasticity in PD as a means for eliciting improvement in motor performance has underscored the importance of identifying those exercise parameters that are essential for promoting activity-dependent neuroplasticity. Findings from our study suggest a potential role of intensity of exercise in driving activity-dependent neuroplasticity and functional improvement in people with PD and warrants further investigation.

To our knowledge, this is the first study to show that people with PD can engage in very high levels of exercise intensity—up to 13.3 METS—and the first to report improvement in both measures of brain and behavior in people with PD as the result of high-intensity exercise. By understanding the effects of exercise on neuroplasticity, novel nonpharmacologic therapeutic modalities may be designed to delay or reverse disease progression in idiopathic PD.

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9 NON-HUMAN PRIMATE MODELS OF PARKINSON'S DISEASE AND EXPERIMENTAL THERAPEUTICS

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INTRODUCTION

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The non-human primate serves as an important model for understanding the pathophysiology of the basal ganglia, evaluating new treatment modalities for neurodegenerative disorders affecting this region, especially Parkinson's disease (PD), and provides a valuable tool for discovery of new therapeutic targets that may lead to a cure for PD. The non-human primate model generated through either neurotoxicant or surgical lesioning has been most commonly used for experimental therapeutic studies in PD, in particular for identifying new symptomatic strategies primarily targeting the dopaminergic system, as well as those neurotransmitter systems known to modulate dopamine (including serotonin, glutamate, adenosine, acetylcholine, endocannabinoid, and noradrenalin). The model has also been extremely valuable in providing important insights into the pathophysiology and treatment of levodopa-induced dyskinesia, a disabling complication of long-term levodopa use in PD. In addition, the model has provided fertile ground for investigating innovative therapeutic approaches such as gene therapy using vector delivery systems, tissue transplantation, neurotrophic factor delivery, and deep brain stimulation (DBS). Finally, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned non-human primate model has been valuable for understanding basal ganglia function in both the normal and lesioned state by providing insights into the role of neurotransmitters and circuitry in motor learning, motor control, and synaptic function that will guide the development of new therapeutic approaches for PD and related disorders (Israel, 2007). The strength AUQ3 of the non-human primate as a model of PD lies in its similarities to the human condition for (i) clinical phenomenology, (ii) response to dopamine replacement therapy, and (iii) neuroanatomy. These strengths have been confirmed by 25 years of investigations that have yielded valuable insights toward the treatment of PD and normal basal ganglia function.

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Although the non-human primate model has been important in identifying many new treatments for PD, including pharmacological targeting of non-dopaminergic neurotransmitter systems, vector-based gene therapy, and tissue transplant approaches, a number of these preclinical studies, did not accurately predict efficacy in clinical trials. There are many factors that may contribute to this lack of a translational success including (i) limitations in our knowledge regarding basal ganglia function in both the normal and diseased state; (ii) limited understanding of the pharmacokinetics and bioavailability of novel compounds; (iii) poorly elucidated adverse effects, including cardiovascular

Parkinson's Disease: molecular and therapeutic insights from model systems
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and cognitive changes; (iv) limited understanding of molecular mechanisms of the treatment intervention; and (v) differences between preclinical and clinical study design. Still, studies have shown that the non-human primate model addresses many important questions in addition to efficacy, including safety and tolerability, and technical issues addressing feasibility. More importantly, the model can be used to understand the underlying molecular mechanisms responsible for the success or failure of any new therapy, and consequently becomes a valuable conduit in bi-directional translational medicine from bench to bedside and bedside to bench. The value of information gained from studies using the non-human primate model of PD resides in understanding the details and differences of any one of the several distinct non-human primate models that may be used in the experimental paradigm. Important parameters that may vary amongst the many different non-human primate models include the lesioning regimen, behavioral assessments, and the pathological, neurochemical and molecular features of the model, some of which may be dynamic and display altered plasticity weeks to months post-lesioning.

p0030 The primary goal of this chapter is to review commonly used non-human primate models for PD, including (i) the behavioral assessments used to measure parkinsonian motor features or levodopa-related abnormal involuntary movements; (ii) the different model types and subtypes; (iii) the use of these models in pharmacological and non-pharmacological experimental therapeutics; and (iv) the impact of these models in understanding basal ganglia function and physiology. While the vast majority of non-human primate models of PD utilize the neurotoxicant MPTP, other neurotoxicants with similar selectivity for midbrain dopaminergic neurons, such as 6-hydroxydopamine, methamphetamine (METH), and proteasome inhibitors have been utilized. The development of many of these primate models has often been preceded by studies in rodents, which provided the foundation and basic understanding of these neurotoxicants. By understanding the details of each model, the investigator can appreciate more fully the individual strengths and limitations of the specific models, and can therefore select the most appropriate model for the question under investigation. This will then lead to improved study design, clearly defined outcome measures, and more comprehensive interpretation of preclinical studies.

ASSESSMENT OF MOTOR BEHAVIOR IN NON-HUMAN PRIMATE MODELS OF PD s0020

p0040 Studies evaluating pharmacological and non-pharmacological agents in non-human primate models require the observation and quantification of motor behavior(s) associated with the parkinsonian state. While the primary motor feature is bradykinesia, these animal models may also demonstrate balance problems, tremor, freezing, and changes in posture. Most models also display the motor complications related to levodopa therapy, specifically dyskinesia (chorea and dystonia), and in some cases wearing-off phenomena. Several approaches have been used to quantify motor activity related to either the parkinsonian state or levodopa-related dyskinesias, including (i) clinical rating scales (CRSs); (ii) cages designed to measure animal movement (typically by having the animal break infrared beams traversing the cage); (iii) personal activity monitors affixed to the animals (often based on a small accelerometer); and (iv) digitized video monitoring systems. Each of these approaches has advantages and disadvantages.

Clinical Rating Scales s0030

p0050 Since the initial reports of the utility of MPTP in non-human primate, a number of different CRSs have been published. These scales vary with respect to the species of the model and behaviors that are readily observed after MPTP administration. The design of a CRS is often developed by comprehensive observations of the full behavioral repertoire of the animal model. Most rating scales are then often refined to highlight those behavioral features that have a human clinical correlate as assessed in the Unified Parkinson's Disease Rating Scale (UPDRS). In general, many of these published scales for the non-human primate models have been tested for inter-rater reliability, validated against other behavioral measures, and have been demonstrated to be sensitive enough to capture response to intervention. For example, in the squirrel monkey, CRSs have been developed that have been shown to be sensitive to the degree of MPTP-lesioning and to capture behavioral features that are responsive to levodopa replacement therapy (Petzinger *et al*., 2001). Rating scales have also been devised to evaluate the presence and intensity of motor complications in a number of different non-human primate models including the squirrel monkey and the marmoset (Petzinger *et al*.,

AUQ4 2001; Tan *et al*., 2002; Jenner, 2003). These scales also vary within the different models. For example some scales distinguish between the types (chorea versus dystonia), and distribution (generalized versus focal) of dyskinesia, while others do not.

p0060 The main disadvantages of CRSs are that their application is often labor-intensive, and rating assignments are subjective and require extensive training. In addition, while many scales attempt to highlight features of animal behavior that resemble human parkinsonism, some behavioral features included in the rating scales are unique to a specific model, and do not have a direct human correlate. For example, action tremor, which is not a classical clinical feature of PD, is frequently observed in the squirrel monkey after MPTP administration and is often included in the clinical rating of these animals. In addition, hypokinesia or a general poverty of movement of an animal in its environment is another behavioral feature that is often observed and rated, but has no direct correlation with the UPDRS rating scale in human PD. The degree to which MPTP-induced behavioral features should be included in a CRS, despite the lack of a direct human clinical correlate, is an issue of debate.

s0040 Automated Behavioral Observation Methods

p0070 Another approach to measuring parkinsonian motor behavior is through the application of activity monitors either applied to the home cage or attached to the primate itself. Cage-based activity monitors are most commonly grounded in technologies similar to those used in rodent studies where movements are counted when a subject breaks infrared beams traversing the cage. These techniques distinguish between repetitive breaking of a single beam and consecutive breaks of different beams, thus allowing the apparatus to distinguish translational movement of the animal through space, which provides a measure generally accepted to represent locomotor activity. A recent technique utilizes telemetric methods to detect a probe implanted in the subcutaneous tissue of the animal to monitor locomotion. Personal activity monitors, such as the small accelerometers manufactured by Minimitter Company (Bend, OR), can be attached to the subject either using a collar, jacket, or straps. Most non-human primates require training or habituation to adapt to these encumbrances. These personal monitors measure general motor activity, so that the output encompasses both translational movement through space and movements performed with the animal stationary in the cage. Distinguishing between the different movement types, however, is impossible from the raw data from this device alone.

p0080 Newer types of activity measures have been derived from the advances in computer-based video technologies. These methods span a wide spectrum. At the low end are ones requiring minimal expense but provide little or no technical support and yield relatively crude measures. In contrast, there are other, commercially produced systems that can track each of the animals' limbs separately, recording position and velocity, and then provide sophisticated analyses of specific movement subtypes, with the trade-off being that acquiring these systems requires substantial financial investment.

p0090 Each of these systems has advantages and disadvantages. Choosing which method to use for measuring activity depends upon the specific requirements of the study design. In many cases, complementary methods are combined. For example, an automated system is often used in conjunction with a CRS. We suspect that, in the future, a popular approach will be the simultaneous application of a video-based computerized method with ratings performed on the video images.

p0100 One possible consideration in selecting a behavioral monitoring method is its ability to be applied repeatedly in a serial fashion to generate a time course. Because behavior is highly variable, the additional data points gathered in constructing a time course allows for much greater power in the statistical analyses. Furthermore, it is often difficult to know *a priori* how long after drug administration the peak effect will occur: making a series of repeated measurements throughout the entire duration of the behavioral change provides a more accurate assessment than making one measurement at a single time point when the peak effect is presumed to occur. This assumes even greater importance in studies using multiple drugs, leading to complex pharmacokinetic interactions. For example, using one drug to suppress levodopa-induced dyskinesias could lead to uncertainty about what aspect of the behavior might be affected: the duration of the dyskinesias might be shortened, the severity of the peak effect might be lessened, or both might occur. Automated methods in home cages have an obvious advantage for making repeated measurements over an extended period of time. In contrast, clinical ratings may be

challenging when performed at cage side for long periods of time. However, clinical rating methods can be applied in conjunction with video recording over an extended period of time with animals in their home cages.

p0110 In contrast, the more objective methods of motor assessment in home cages using mechanical (accelerometers) and electronic (infrared beams) technology provide quantitative data on movement in space that can undergo parametric statistical analyses. However, these methods do not often target different subtypes of movement, or specific body regions. The more recent video-based systems with computerized analysis can have advantages over both clinical and automatic cage monitors by providing sophisticated quantitative analyses while simultaneously distinguishing different types of movements.

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Data Analysis

p0120 Behavioral experiments tend to produce large amounts of data requiring thorough and sometimes complex statistical analysis. These analyses can be performed using currently available statistical software packages, such as SPSS (SPSS Inc., Chicago, IL) or SAS/STAT (SAS America Inc., Cary, NC). For example, the statistical analysis using time as a variable requires the application of repeated measures analysis of variance for parametric variables, but may require more sophisticated analysis for nonparametric variables (Togasaki *et al*., 2005). Some studies may require correlation analysis to compare one behavioral analysis to another such as the improvement in levodopa-induced dyskinesias compared with the change in the severity of parkinsonism (Hsu *et al*., 2004). Researchers can benefit from the inclusion of statisticians as a component of a study not only for the analysis of data but also for the early stages of the experimental design.

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NON-MOTOR FEATURES IN MPTP-LESIONED NON-HUMAN PRIMATES

p0130 A potentially valuable application of the MPTP-lesioned non-human primate is in elucidating the anatomical sites and neurotransmitter systems involved in non-motor behavior. Although the substantia nigra pars compacta (SNpc) is the most vulnerable region affected by MPTP, there are varying degrees of injury and/or cell death in other brain areas including the ventral tegmental area (VTA), retrorubal field, nucleus Basalis of Meynert, raphe nucleus, and locus ceruleus (Mitchell *et al*., 1985a; Forno, 1986a, b). The fact that these anatomical regions mediate non-motor behavior and that MPTP can alter their neurotransmitter systems (including serotoninergic, cholinergic, and noradrenergic) suggests that non-motor features may be observed in these models (Namura *et al*., 1987; Friedman and Mytilineou, 1990; Perez-Otano *et al*., 1991). Unfortunately, there have been a limited number of studies in the MPTP-lesioned non-human primate examining non-motor features, which include cognitive impairment (executive function), sleep disorders, and affective behaviors, such as depression and anxiety (Schneider and Pope-Coleman, 1995; Decamp and Schneider, 2004). For example, cognitive changes, including reduced executive function and attention, are evident with early parkinsonian features after chronic low-dose MPTP administration, which might be associated with injury of extrastriatal dopaminergic pathways to the prefrontal cortex (Schneider, 1990; Schneider and Kovelowski, 1990; Schneider *et al*., 1995; Slovin *et al*., 1999a, b; Decamp and Schneider, 2004). In addition, other cognitive deficits in working memory, which might reflect dopaminergic injury to the hippocampus, can be observed (Decamp *et al*., 2004). Alterations in sleep organization patterns have also been observed in MPTP-lesioned monkeys, with more severe disturbances in animals receiving a greater amount of MPTP (Almirall *et al*., 1999).

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THE MPTP-LESIONED NON-HUMAN PRIMATE MODEL

p0140 The most common model of PD in the non-human primate is generated by the administration of the neurotoxicant MPTP. The major strengths of this model include (i) parkinsonian features that closely resemble the human condition; (ii) the robust response to dopaminergic therapy; (iii) manifestation of levodopa-induced motor complications including dyskinesia and wearing-off; and (iv) neuroanatomical and physiological features that are similar to that in humans. This model has been well validated over the last 25 years as reflected in its ability to successfully predict the efficacy of drug treatments in patients with PD. In the following sections we present the history of MPTP, features of the MPTP

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non-human primate model, the different subtypes of models generated using various lesioning regimens, and finally, examples of the utility of the model for evaluating new therapeutic agents and strategies, and for providing insights into basal ganglia function in the normal and parkinsonian states.

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A BRIEF RETROSPECTIVE OF MPTP IN MONKEYS

p0150 Immediately following its identification in humans, MPTP was administered to both rodents and non-human primates and some of the most valuable animal models of PD were established. MPTP-lesioned non-human primate models encompass a wide spectrum of species including the squirrel monkey (*Saimiri sciureus*) (Langston *et al*., 1984), long-tailed macaque or cynomologus (*Macaca fascicularis*) (Mitchell *et al*., 1985), rhesus macaque (*Macaca mulatta*) (Burns *et al*., 1983; Chiueh, 1984b; Markey *et al*., 1984), Japanese macaque (*Macaca fuscata*) (Crossman *et al*., 1985; Jenner *et al*., 1986), bonnet monkey (*Macaca radiata*) (Freed *et al*., 1988), owl monkey (*Aotus trivirgatus*) (Collins and Neafsey, 1985), baboon (*Papio papio*) (Hantraye *et al*., 1993; Moerlein *et al*., 1986), African green monkey or vervet (*Chlorocebus aethiops* formerly *Ceropithecus aethiops*) (Taylor *et al*., 1994), pigtail macaque (*Macaca nemestrina*) (Chefer *et al*., 2007), and common marmoset (*Callithrix jacchus*) (Jenner *et al*., 1986). The administration of MPTP to the non-human primate results in parkinsonian symptoms including bradykinesia, postural instability, freezing, stooped posture, and rigidity. Although postural and action tremors have been observed in many species after MPTP treatment, a resting tremor, characteristic of PD, is less commonly documented (Tetrud *et al*., 1986; Hantraye *et al*., 1993; Raz *et al*., 2000).

p0160 The mechanism of MPTP toxicity has been thoroughly investigated. The meperidine analog MPTP is converted to 1-methyl-4-pyridinium (MPP^+) by monoamine oxidase B. MPP^+ acts as a substrate of the dopamine transporter (DAT) and is selectively taken up by the dopaminergic cells of the SNpc, leading to the inhibition of mitochondrial complex I, the depletion of ATP, and cell death. In mice and non-human primates MPTP selectively destroys dopaminergic neurons of the SNpc, the same neurons affected in PD (Langston *et al*., 1984; Forno *et al*., 1993; Jackson-Lewis *et al*., 1995). Similar to PD other catecholaminergic neurons, such as those in the VTA and locus coeruleus, may be affected albeit to a lesser degree (Mitchell *et al*., 1985a; Forno, 1986b; Forno, 1996). In addition, dopamine depletion occurs in both the putamen and caudate nucleus. Whether the putamen or caudate nucleus is preferentially lesioned may depend on animal species and regimen of MPTP administration (Ricaurte *et al*., 1986; Kalivas *et al*., 1989; Bezard *et al*., 2000).

p0170 Unlike PD, Lewy Bodies have not been consistently documented. Animal age and route of MPTP administration are two factors that may influence the development of Lewy Bodies. Specifically, eosinophilic inclusions (resembling Lewy Bodies) have been described in aged MPTP-lesioned squirrel and cynomologus monkeys (Forno, 1986a; Kiatipattanasakul *et al*., 2000). In humans, autosomal recessive juvenile parkinsonism (AR-JP) due to mutations in the *parkin* gene do not develop Lewy Bodies (Shimura *et al*., 2000) whereas Lewy Bodies are common (and serve as the pathological hallmark) in idiopathic PD in the aged brain (Jellinger, 2001). The time course of MPTP-induced neurodegeneration is rapid, and therefore, represents a major difference from idiopathic PD, which is a chronic progressive disease manifesting over several years. Modifications in the MPTP-lesioning regimen, such as chronic treatment in the presence of probenecid or the delivery of alpha-synuclein via stereotactic targeting of expression vectors have been reported to promote the formation of intracellular protein aggregates reminiscent of Lewy Bodies (Meredith *et al*., 2002; Eslamboli *et al*., 2007). The requirement of Lewy Body formation in animal models is an issue of debate since they might be neurotoxic or they might be neuroprotective (Calne *et al*., 2004; Bodner *et al*., 2006).

p0180 Following the administration of MPTP, the non-human primate progresses through acute (hours), sub-acute (days), and chronic (weeks) behavioral phases of toxicity that are due to the peripheral and central effects of MPTP. The acute phase occurs within minutes after MPTP administration and is characterized by sedation, and a hyper-adrenergic state. This state may also include hyper-salivation, emesis, exaggerated startle, seizure-like activity, and dystonic posturing of trunk and limbs (Jenner *et al*., 1986; German *et al*., 1988; Jenner and Marsden, 1988; Petzinger and Langston, 1998; Irwin *et al*., 1990). The sub-acute phase generally occurs within hours and persists for several days and may be due to the peripheral actions of MPTP on the autonomic

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nervous system and peripheral organs such as the liver, kidney, and heart (see below) (Petzinger and Langston, 1998). Weight loss, altered blood pressure, and hypothermia may occur, requiring oral gastoral tube feeding and placement in an incubator to stabilize body temperature. In addition, elevated liver transaminases and creatinine phosphokinase may develop; reflecting impaired liver function and muscle breakdown. Behaviorally, these animals may appear prostrate and cognitively impaired. Occasionally, animals may demonstrate self-injurious behavior such as finger biting and hyper-flexion of the neck and trunk with head banging. Assessment of parkinsonian features may be confounded by alterations in the general health of the animal. The chronic phase starts within days to weeks after MPTP administration. It is characterized by the stabilization of body weight and temperature as well as the normalization of blood chemistries such as hepatic enzymes. Parkinsonian features clearly emerge and remain stable for weeks to months or longer. Similar to PD, the MPTP-lesioned non-human primate responds to traditional anti-parkinsonian therapies such as levodopa and dopamine receptor agonists; in some cases severely lesioned animals may require such an intervention to sustain survival. The degree of behavioral stability may be predicted in part by the initial degree of behavioral impairment as observed between the subacute and the chronic phases. Animals with greater behavioral impairments require a longer period of time for recovery. Behavioral improvement after MPTP administration has been reported in most species of non-human primates (see below). In general the behavioral response to MPTP-lesioning may vary both within and between species. One must be cautious in simply extrapolating findings from one species or strain to another. For example, many, but not all, strains of mice are sensitive to the effects of MPTP, whereas rats are almost completely resistant (Riachi, 1988; Zuddas *et al*., 1994). This variability due to age and species phylogeny also applies to the non-human primate. For example, Old World monkeys (such as rhesus, *Macaca mulatta* or African Green, *Chlorocebus aethiops*) tend to be more sensitive to MPTP administration than New World monkeys (such as the squirrel monkey, *Saimiri sciureus* or marmoset, *Callithrix jacchus*) (Rose *et al*., 1993; Ovadia *et al*., 1995; Gerlach and Reiderer, 1996). Also, within a species, younger animals tend to be more resistant to the effects of MPTP and are more likely to recover from mild lesions compared to older animals (Ovadia *et al*., 1995; Collier *et al*., 2007). There are a number of different reasons that account for the variability in MPTP sensitivity including its metabolism (especially in the liver), ability to cross the blood–brain barrier, conversion of MPTP to the toxic form MPP^+ in the brain primarily by astrocytic monoamine oxidase B, uptake into dopaminergic neurons through the DAT, and distribution within cells to target mitochondrial energy depletion and to mediate cell death (Dauer and Przedborski, 2003; Jackson-Lewis and Smeyne, 2005).

THE SUBTYPES OF THE MPTP-LESION NON-HUMAN PRIMATE MODEL

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Administrating MPTP through a number of different regimens has led to the development of several distinct models of parkinsonism in the non-human primate that vary in both behavioral and pathological features. Each model is characterized by unique behavioral and neurochemical features, which should be taken into consideration when addressing specific scientific objectives or designing studies. As a result, numerous studies addressing a variety of hypothesis have been carried out in these different models. These studies include the investigation of basal ganglia function including motor behavioral recovery, mechanisms of motor complications, and cognitive impairment. These models also help in the evaluation of new treatment modalities including pharmacological agents, cell transplantation, DBS, and novel neuroprotective and restorative strategies. In some models (such as the intracarotid delivery of MPTP) there is profound striatal dopamine depletion and denervation with few or almost no dopaminergic axons or terminals remaining. This model provides an optimal setting to test fetal tissue grafting since the presence of any tyrosine hydroxylase positive axons or sprouting cells would be due to surviving transplanted tissue or its influence on intrinsic striatal neurons. Other models, such as mild systemic delivery of MPTP, have less extensive dopamine depletion and only partial denervation with a moderate number of dopaminergic axons and terminals remaining. This partially denervated model best resembles mildly to moderately affected PD patients. Therefore, sufficient dopaminergic neurons and axons as well as compensatory mechanisms are likely to be present. Growth factors (inducing sprouting) or neuroprotective factors

(promoting cell survival) are best evaluated in this situation. The most commonly used MPTP-lesioning paradigms in non-human primate models include (1) the systemic-lesioned model, (2) hemi-lesioned, (3) bilateral intracarotid, (4) over-lesioned, and (5) low-dose chronic. The following sections briefly highlight the features of these different models. It should be kept in mind that these models are delineated based on the mode of MPTP delivery rather than the species of non-human primate utilized. Other factors will influence these models due to the degree of lesioning including the age, subspecies, and sex of the animals used.

o0010 (1) In the *systemic-lesioned model*, MPTP is administered via intramuscular, intravenous, intraperitoneal, or subcutaneous injection (Waters *et al*., 1987; Eidelberg *et al*., 1986; Tetrud and Langston, 1989; Elsworth *et al*., 1990). This model is most common with smaller non-human primates such as the squirrel monkey and marmoset, which can tolerate modest levels of MPTP. Old World monkeys tend to display a high degree of sensitivity toward MPTP and if utilizing systemic administration small doses are typically used and are spread out over several weeks. Large doses result in severe akinesia and may require intensive veterinarian intervention including prolonged hand feeding to rescue animals. In addition, large doses can result in increased risk of animal death from the effects of MPTP and MPP^+ on peripheral organs. Systemic administration of MPTP leads to bilateral depletion of striatal dopamine and nigrostriatal cell death. One feature of this model is that the degree of lesioning can be titrated by adjusting the MPTP concentration administered resulting in a range (mild to severe) of parkinsonian symptoms. The presence of clinical asymmetry in motor features is common with one side more severely affected, but this feature may be subtle. Levodopa or dopamine agonist administration leads to the reversal of all behavioral signs of parkinsonism in a dose-dependent fashion. After several days to weeks of levodopa administration, animals develop reproducible motor complications. The principal advantage of this model is that the behavioral syndrome closely resembles the clinical features of idiopathic PD. The systemic model has partial dopaminergic denervation bilaterally and probably best represents the degree of loss seen in all stages of PD including end-stage disease, where some dopaminergic neurons are still present. This model is well suited for therapeutics that interact with remaining dopaminergic neurons including growth factors, neuroprotective agents, and dopamine agonists. The easily reproducible dyskinesia in this model allows for extensive investigation of its underlying mechanism and treatment (see below). Disadvantages of this model include spontaneous recovery in mildly affected animals, while severely affected animals may require extensive veterinary care and dopamine supplementation, specifically in the early period following MPTP-lesioning. In most cases early interventive care is necessary to overcome the systemic effects of MPTP on peripheral organ systems including the liver, kidneys, and heart which are typically transient (see section on systemic effects of MPTP below).

o0020 (2) The *hemi-parkinsonian or hemi-lesioned model* involves administration of MPTP via unilateral intracarotid infusion and has been used to induce a hemi-parkinsonian state in the primate (Bankiewicz *et al*., 1986). The surgical delivery of MPTP goes directly to the brain, avoiding the systemic effects of MPTP as well as potential xenobiotic metabolism in the liver influencing its bioavailability. The rapid metabolism of MPTP to MPP^+ in the brain may account for the localized toxicity to the hemisphere ipsilateral to the infusion and in some cases can induce stroke-like lesions and necrosis within the basal ganglia (Emborg *et al*., 2006). Motor impairments appear primarily on the contralateral side. Hemi-neglect, manifested by a delayed motor reaction time, also develops on the contralateral side. In addition, spontaneous ipsilateral rotation may develop. Levodopa administration reverses the parkinsonian symptoms and induces contralateral rotation. SNpc neurodegeneration and striatal dopamine depletion (greater than 99%) on the ipsilateral side to the injection is more extensive than in the systemic model. The degree of unilateral lesioning in this model is dose-dependent. Major advantages of this model include (i) the ability of animals to feed and maintain themselves without supportive care; (ii) the availability of the relatively unaffected limb on the ipsilateral side to serve as a control; and (iii) the utility of the dopamine-induced rotation for pharmacological testing. In addition, due to the absence of dopaminergic innervation in the striatum, the hemi-lesioned model is well suited for examining neuronal sprouting of transplanted cells or tissue. A disadvantage of this model is that only a subset of parkinsonian features are evident and are restricted to one side of the body, a situation never seen in idiopathic PD.

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o0030 (3) The *bilateral intracarotid model* employs an intracarotid injection of MPTP followed several months later by another intracarotid injection on the opposite side (Smith *et al*., 1993). This model combines the less debilitating features of the carotid model with bilateral clinical features, a situation more closely resembling idiopathic PD. The advantage of this model is its prolonged stability and limited inter-animal variability. Similar to the hemi-lesioned model, where there is extensive striatal dopamine depletion and denervation, the bilateral intracarotid model is well suited for evaluation of transplanted tissue or vector infusion. However, levodopa administration may result in only partial improvement of parkinsonian motor features and food retrieval tasks. This can be a disadvantage since high doses of test drug may be needed to demonstrate efficacy, increasing the risk for medication-related adverse side effects.

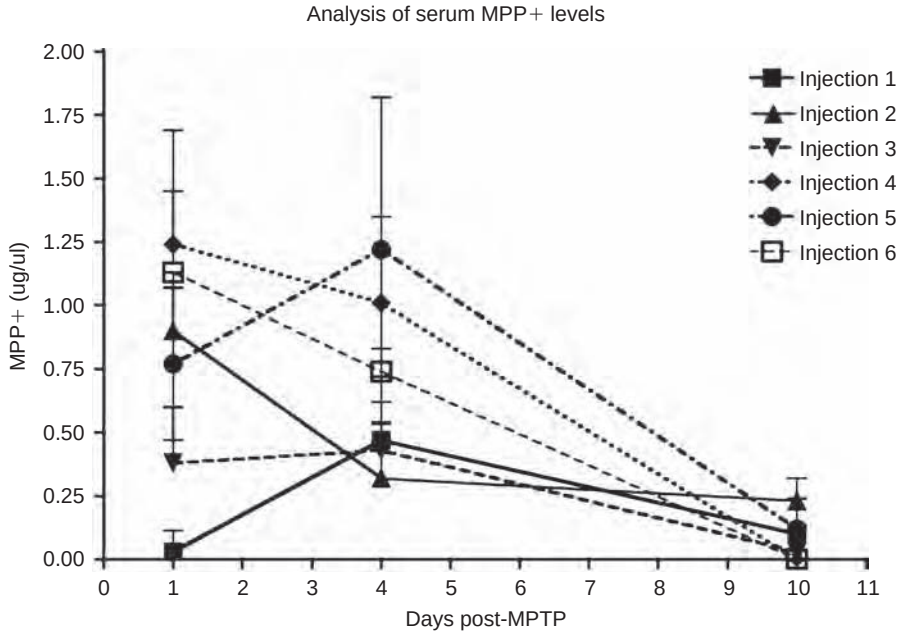
o0040 (4) The *over-lesioned model* is a novel approach to MPTP-lesioning and involves the administration of MPTP via intracarotid infusion followed by a systemic MPTP injection (Eberling *et al*., 1998). This model is characterized by severe dopamine depletion ipsilateral to the MPTP-carotid infusion and a partial depletion on the contralateral side due to the systemic MPTP injection. Consequently, animals are still able to maintain themselves as they retain one relatively intact side. The behavioral deficits consist of asymmetric parkinsonian features. The more severely parkinsonian side is contralateral to the intracarotid injection. Levodopa produces a dose-dependent improvement in behavioral features. The complications of levodopa therapy, however, such as dyskinesia have not been as consistently observed. This model combines some of the advantages of both the systemic and intracarotid MPTP models, including stability. This model is suitable for both transplant studies, utilizing the more depleted side, and neuro-regeneration with growth factors, utilizing the partially depleted side where dopaminergic neurons still remain.

o0050 (5) The *chronic low dose model* consists of intravenous injections of very low dose of MPTP administration over a 5-to 13-month period (Bezard *et al*., 1997; Slovin *et al*., 1999a, b; Decamp and Schneider, 2004). This mode will be discussed in details in chapter of this book. Rather than primarily targeting motor behavior this model is characterized by cognitive deficits consistent with frontal lobe dysfunction reminiscent of PD or normal-aged monkeys. These animals have impaired attention and short-term memory processes and perform poorly in tasks of delayed response or delayed alternation. Since gross parkinsonian motor symptoms are essentially absent at least in the early stages, this model is well adapted for studying cognitive deficits analogous to those that accompany idiopathic PD.

THE ADVERSE EFFECTS OF MPTP-LESIONING s0100

p0250 In addition to its motor effects, MPTP clearly has additional central nervous system effects that may include hypothermia and seizures. The systemic administration of MPTP may also lead to deleterious peripheral effects especially on systemic organs that must be taken into consideration when designing therapeutic studies since they could lead to adverse effects influencing animal behavior, alterations in drug bioavailability, or alterations in drug–target interactions. For example, the peripheral conversion of MPTP to MPP^+, especially in the liver, can result in short-term changes in liver metabolism. Alterations within the liver can influence subsequent MPTP administration sessions (especially those occurring within the first week) and may alter xenobiotic metabolism of MPTP, MPP^+, or the therapeutic drug of interest. This point is illustrated in Figure 9.1. The level of serum MPP^+ was determined by HPLC analysis after each of six successive AUQ5 subcutaneous MPTP injections at 2-week intervals in the squirrel monkey. Data collected at post-lesioning day 1, 4, and 10 after each injection demonstrates altered serum MPP^+ levels indicating that the peripheral conversion of MPTP to MPP^+ is occurring in a successively higher rate with each injection. Since MPP^+ itself does not cross the blood–brain barrier, there is subsequent reduced bioavailability of the toxin to the brain and therefore less MPTP-mediated cell death. Our analysis of brain tissue early in the injection regimen suggests that the majority of MPTP-induced cell loss of midbrain dopaminergic neurons occurs within the period of the first three injections.

p0260 Systemic insult in the non-human primate may also influence motor and non-motor behavior, independent of the central brain lesion itself, since animals that are sick due to MPTP may become very quiescent and disengaged. Furthermore, the peripheral effects on systemic organs, especially the heart, kidneys, and liver, are often responsible for the



f0010 **FIGURE 9.1** Altered levels of serum MPP^+ levels with successive injections of MPTP. Squirrel monkeys were administered a series of six injections of MPTP (i.p., 2.0 mg/kg free-base, 2 weeks between each injection). Blood was collected at days 1, 4, and 10 after each injection of MPTP and the level of MPP^+ determined by HPLC analysis. These data demonstrate that with successive injections of MPTP there is an increase in the serum level of MPP^+ indicating systemic conversion of MPTP to MPP^+ most likely due to induction of metabolic enzymes in peripheral organs especially the liver. Since MPP^+ cannot cross the blood–brain barrier the degree of lesioning in the brain is reduced with later injections of MPTP.

death of animals following MPTP administration. Death can occur within hours of MPTP administration indicating an immediate organ failure, or within the first week due to the inability of animals to eat or drink for themselves. In addition, animals can become cachectic, and can show general wasting and decline without proper intervention. Supportive intervention to avoid this adverse effect includes feeding lesioned non-human primates by gavage with an enriched diet, injection of subcutaneous fluids, or the administration of levodopa to promote movement. Investigators must be cognizant of using dopamine replacement therapy for rescue treatment since such intervention may influence experimental outcome measures.

p0270 To address the potential adverse effects of MPTP, we studied issues of the systemic effects of MPTP to gain insight into interventions that would result in greater animal survival. Squirrel monkeys were administered MPTP (in a series of six subcutaneous injections of 2 mg/kg, free-base, 2 weeks apart) and were given a comprehensive physical examination 1, 4, and 10 days after each injection. The results are summarized in Table 9.1. After the first injection of MPTP, there were significant alterations in a number of physiologic parameters including elevated liver transaminases and elevated creatinine phosphokinase, indicative of liver damage and muscle breakdown, respectively. By the second injection, there was a significant decrease in body weight, which was cumulative with each subsequent injection and tended not to recover without intervention (gavage feeding). Greater than 20% loss of body weight is a significant predictor of animal death. Evidence of hepatocellular toxicity persisted for several weeks after the final MPTP injection. In addition, animals had hypothermia beginning 48 h after lesioning and persisting for up to 10 days

t0010 **TABLE 9.1 Systemic effects of MPTP in the non-human primate. Squirrel monkeys received a series of six injections of MPTP (s.c., 2.0 mg/kg, free-base) with 2 weeks between injections. At days 1, 4, and 10 after each of the six MPTP injections animals were subjected to a comprehensive physical exam that included body weight, heart rate, blood pressure, core body temperature, blood cell counts, and comprehensive blood chemistry. Data are grouped according to either the MPTP exposure early (injections 1–3) or late (injections 4–6) as well as post-lesion time early (1 day) and late (10 days). These data demonstrate the variation in systemic parameters at different stage of lesioning**

Measures	Change			
	Time		Injection #	
	Early	Late	Early	Late
Cardiovascular				
Heart rate	↑	↓	…	…
Blood pressure	↓	↓	↓	↓
Body weight	↓	↓	↓	↓
Body temperature	↓	↑	↓	↓
Blood cell counts				
White blood Cells	↑	↓	↓	↑
Hemoglobin	↓	↓	↓	↓
Reticulocytes	↑	↓	↓	↑
Hematocrit	↓	↓	↓	↓
Blood chemistry				
Creatine phosphokinase	↑	↑	↑	↑
Alanine transaminase	↑	↑	↑	↑
Aspartate transaminase	↑	↑	↑	↑
Alkaline phosphatase	↑	↑	↑	↑
Creatine	…	…	…	…
Blood urea nitrogen	↓	↓	↓	↓
Sodium	…	…	…	…
Potassium	…	↑	…	↓
Chloride	…	…	…	…

Early time = Day 1; Late time = Day 10; Early injection = 1, 2, 3; Late injection = 4, 5, 6

↑ = Generally increased compared to baseline

↓ = Generally decreased compared to baseline

… = Similar to baseline

after the last MPTP injection. The pathophysiology of these effects may be directly related to MPTP itself and/or its metabolites and their adverse effects may persist for several weeks. Overall, body weight and white blood cell count were the key predictors of mortality and should be monitored during MPTP administration. Supplemental caloric intake may be helpful in improving survival. These studies highlight the systemic effects of MPTP on animal models that should be taken into consideration during the design of any pharmacological study. Some researchers may be interested in details of MPTP toxicity and safety as it pertains to risks of exposure to researchers. Using good laboratory practice and general safety procedures MPTP can be utilized with little risk. Researchers may refer to

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specific technical reviews for additional information (Przedborski *et al*., 2001; Jackson-Lewis and Przedborski, 2007)

s0110

TESTING PHARMACOLOGICAL THERAPIES FOR NEUROPROTECTIVE AND SYMPTOMATIC BENEFIT

p0280

The MPTP non-human primate model can be used to test compounds that may provide neuroprotective or symptomatic benefit. In neuroprotective studies, it is extremely valuable to understand the mechanism(s) and time course of toxin-induced nigrostriatal dopaminergic cell death. This information is critical in determining the timing of drug administration relative to toxin exposure, and in providing possible explanations to neuroprotection that may include toxin bioavailability. Neuroprotective interventions are typically started before or during the lesioning phase, and may therefore vary depending on the animal species and timing of toxin-induced cell death. For example, in the non-human primate, such as the squirrel monkey, the half-life of MPTP/MPP^+ clearance is approximately 11 h (Irwin, 1985; Irwin *et al*., 1990). In contrast, the half-life of MPTP/MPP^+ clearance in the C57BL/6 mouse is approximately 3 h and the time course of nigrostriatal dopaminergic neuron cell death is complete by day 3 post-injection (Irwin, 1989; Jackson-Lewis *et al*., 1995). Drug-related neuroprotection study may be due to the attenuation of cell death by directly supporting midbrain dopaminergic neurons to promote survival (as seen with neurotrophic factors), or it may be due to reduced insult to the brain by altering the bioavailability of toxin. For example, mechanisms by which a drug may effect MPTP/MPP^+ bioavailability include (i) inhibition of monoamine oxidase B, which would suppress the conversion of MPTP to MPP^+ and (ii) alterations in the pattern of expression of proteins involved in MPP^+ uptake and storage, such as the DAT and vesicular monoamine transporter-2 (VMAT-2), respectively (Gainetdinov, 1997; Staal, 2000). Finally, given that many species of non-human primates demonstrate spontaneous behavioral recovery after MPTP administration, it is important to include lesion-only control animals to assess and compare behavioral recovery that is intrinsic to the model versus that due to the neuroprotective agent (Elsworth, 1989; Petzinger *et al*., 2006).

AUQ6 AUQ7 AUQ8 AUQ9

p0290

The MPTP-lesioned non-human primate model is especially useful for evaluating new symptomatic treatments of PD. The primary utility of the model has been to test compounds for symptomatic relief of motor deficits, including bradykinesia, balance impairment, and freezing. Because cognitive and affective behaviors in the MPTP-lesioned non-human primate have not yet been well characterized, and have limited scales to evaluate treatment, few studies have targeted non-motor features in this model. The testing of symptomatic drug therapies often involves the use of an experimental therapeutic colony of non-human primates that have been rendered parkinsonian after MPTP administration, and have been followed for behavioral stability for weeks to months. The experimental therapeutic colony may undergo testing of numerous and a diverse list of symptomatic therapy that may include drugs that directly or indirectly affect dopamine neurotransmission. These drugs include compounds that act directly on dopamine receptor subtypes within the striatum, including dopamine agonists, or compounds that target other neurotransmitter systems such as adenosine or glutamate that may enhance dopamine neurotransmission either directly (by acting on dopamine receptors themselves) or indirectly (by affecting other dopaminergic parameters such as release or downstream effector pathways). Given the repetitive use of the experimental colony in drug testing, investigators must be cognizant of potential long-lasting effects due to drug exposure that could influence subsequent studies.

p0300

The MPTP-lesioned non-human primate has also been valuable in testing potential neurorestorative therapies in PD. The goal of neurorestoration is to re-establish basal ganglia function and improve behavior. Neurorestorative studies include gene/protein delivery via stereotactic targeting or transplantation of genetically engineered or stem/progenitor cells. The different MPTP-lesioned non-human primate models provide contrasting templates to evaluate such interventions. For example, the systemic lesion model typically results in a residual degree of midbrain dopaminergic neuron survival and a partial degree of striatal innervation (Petzinger *et al*., 2006), that may serve as a template to test neurotrophic factors including glia-derived neurotrophic factor (GDNF) (Kordower *et al*., 2000; Oiwa *et al*., 2006). In contrast, the intracarotid lesion paradigm, analogous to the 6-OHDA-lesioned rat, has a near complete depletion of nigrostriatal dopaminergic neurons and their axonal projections. In this

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model, the lesioned basal ganglia may serve as a "blank slate" where the recovery of any dopamine function may be attributed directly to the transplantation of stem/progenitor cells (Taylor *et al*., 1991; Sortwell *et al*., 1998). Specific details of different neurorestorative therapeutic approaches can be found in other related chapters in this book.

p0310 The MPTP-lesioned non-human primate model can also help us better understand the potential properties of pharmacological treatments already in clinical use. For example, an interest in our laboratory is to elucidate the underlying mechanisms of intrinsic motor recovery in the squirrel monkey following systemic lesioning with MPTP (Petzinger *et al*., 2006). It is hypothesized that dopamine could, in fact, act as a neurotrophic factor helping to maintain the integrity of the basal ganglia (Borta and Hoglinger, 2007). We are interested in knowing if dopamine replacement therapy with either levodopa or a dopamine agonist could provide benefit beyond purely symptomatic improvement. In a set of experiments performed in our labs, MPTP-lesioned squirrel monkeys treated with the dopamine agonist pramipexole, and to a lesser extent those treated with levodopa, had higher levels of striatal dopaminergic markers including dopamine and tyrosine hydroxylase, and amphetamine-induced dopamine release than parkinsonian animals treated with saline alone. This occurred despite similar degrees of cell loss based on SNpc counts. These data suggest that dopamine replacement therapy may have a beneficial effect not only on symptomatic treatment of parkinsonian features but also may influence neuroplasticity in the injured basal ganglia. Pharmacological treatment of motor symptoms targeting dopamine replacement may have an analogous effect in patients with PD.

s0120

DYSKINESIA AND MOTOR COMPLICATIONS IN NON-HUMAN PRIMATES

p0320 In PD, levodopa therapy in many patients leads to the development of motor complications, typically after a few years. The underlying pathogenesis of these complications remains obscure (Vitek and Giroux, 2000; Blanchet *et al*., 2004; Brotchie, 2005). For levodopa-induced dyskinesias, abnormal involuntary movements induced by levodopa, studies have implicated several neurotransmitter systems, especially the dopaminergic and the glutamatergic systems. As in patients with PD, the parkinsonian non-human primate also develops complications of levodopa therapy, with both motor fluctuations (wearing-off) and levodopa-induced dyskinesias. In this model, animals develop movements that are abnormal (i.e., they differ phenomenologically from movements that are typically present), but it is impossible to determine whether they are involuntary since we cannot ask the animal its intent in making the movements. We can only judge whether the movements appear purposeful and use this to infer the degree of voluntary control. In any case, the movements bear a striking resemblance to levodopa-induced dyskinesias observed in patients with PD. For example, dyskinetic movements in the MPTP-lesioned squirrel monkey or marmoset involve all four limbs and the trunk, with choreoathetoid movements that develop a few minutes after a dose of levodopa and last 3–4 h. The time course for the movements corresponds to the time course for reversal of MPTP-induced bradykinesia. Observation of the phenomenology of the movements has led to rating scales that have been developed by a number of different groups for squirrel monkeys, marmosets, and macaques (Brotchie and Fox, 1999; Petzinger *et al*., 2001; Chassain *et al*., 2001; Tan *et al*., 2002).

p0330 The effect of pharmacologic agents upon levodopa-induced dyskinesias have been studies for a variety of agents, including D2 dopamine receptor agonists (Calon *et al*., 1995; Smith *et al*., 2006), D3 dopamine receptor partial agonists (Hsu *et al*., 2004; Smith *et al*., 2006), dopamine receptor antagonists (Andringa *et al*., 1999), A2A-adenosine receptor antagonists (Kanda *et al*., 1998; Blandini, 2003), opioid receptor agents (Fox *et al*., 2002; Samadi *et al*., 2003; Samadi *et al*., 2004; Cox *et al*., 2007), and glutamate receptor antagonists (Papa and Chase, 1996; Verhagen-Metman *et al*., 1998; Samadi *et al*., 2007). Unfortunately, the search for an effective suppressor of levodopa-induced dyskinesias has had limited success, although some studies have provided clues for a useful therapeutic strategy; for example, minimizing wide fluctuations in the delivery of levodopa to the striatum. Although the underlying mechanism of levodopa-induced dyskinesias is unknown, electrophysiological, neurochemical, molecular, and neuro-imaging studies in non-human primate models suggest that the pulsatile delivery of levodopa may lead to a variety of changes in the post-synaptic cell and in other regions of the basal ganglia that are further downstream, giving rise to levodopa-induced dyskinesias. These changes could include (i) changes

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in the neuronal firing rate and pattern of the globus pallidus and subthalamic nucleus (STN); (ii) enhancement of D1 and/or D2 dopamine receptor mediated signal transduction pathways; (iii) super-sensitivity of the D2 receptor; (iv) alterations in the phosphorylation state or subcellular localization of glutamate receptors; (v) modifications in dopamine receptor subtypes and their functional links; and (vi) enhancement of opioid-peptide-mediated neurotransmission (Bedard *et al*., 1992; Papa and Chase, 1996; Bezard *et al*., 2001; Hurley *et al*., 2001; Calon *et al*., 2002).

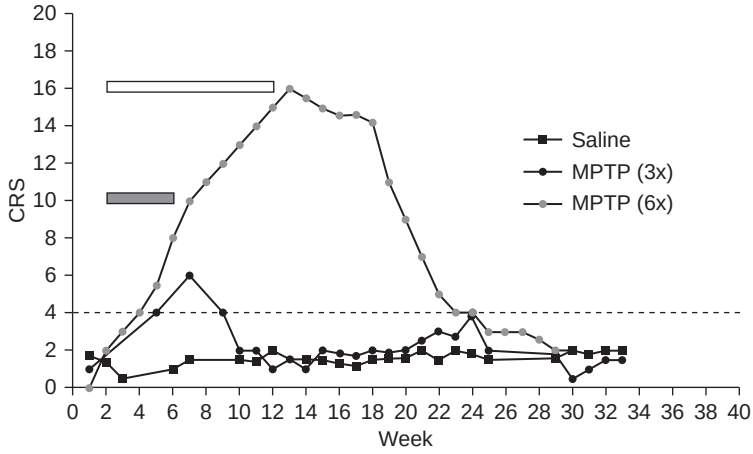
p0340 In designing studies to examine potential treatments for suppressing levodopa-induced dyskinesias, it is necessary to examine the effect on both the dyskinesias and the parkinsonism, as mentioned above. A drug that improves dyskinesias at the expense of worsening parkinsonism will be of limited utility as a treatment in patients. It is also important to keep in mind that dyskinesias differ phenomenologically and possibly mechanistically in different primate species. For example, such a difference might exist for the occurrence of facial dyskinesias, which have been observed in Old World monkeys but not in New World monkeys (Petzinger *et al*., 2001; Tan *et al*., 2002).

p0350 The presence of a nigral lesion has long been considered a necessary prerequisite for the development of levodopa-induced dyskinesias with the intensity dependent on the degree of lesioning (Di Monte *et al*., 2000; Schneider *et al*., 2003; Kuoppamaki *et al*., 2007). Recent studies have challenged this dogma and it has been reported that non-human primates without any dopaminergic lesions can manifest levodopa-induced dyskinesias. For example, when administered sufficiently large doses of levodopa, dyskinesias can develop in squirrel monkeys within a few days (Togasaki *et al*., 2001), and in marmosets within 8 weeks (Pearce *et al*., 2001). The relatively high doses of levodopa administered to these animals may serve to exhaust the buffering capacity of the dopaminergic terminals within the striatum and accentuate the pulsatile deliver of dopamine to the post-synaptic receptors of the normal animal, thus giving rise to dyskinesias.

s0130 INTRINSIC NEUROPLASTICITY AND BEHAVIORAL RECOVERY

p0360 Understanding the molecular mechanisms underlying behavioral recovery in the non-human primate may provide insights into neuroplasticity of the brain after injury, help identify new therapeutic targets for treatment of PD, and provide an opportunity to elucidate basal ganglia function (Zigmond *et al*., 1990; Zigmond, 1997; Bezard and Gross, 1998; Jakowec *et al*., 2003; Jakowec *et al*., 2004). Behavioral recovery after MPTP-induced parkinsonism has been reported in both New World and Old World non-human primates (Eidelberg *et al*., 1986; Kurlan *et al*., 1991; Scotcher *et al*., 1991; Schneider *et al*., 1994, 1995; Cruikshank and Weinberger, 1996; Petzinger and Langston, 1998; Oiwa *et al*., 2003; Petzinger *et al*., 2006). The degree and time course of behavioral recovery is dependent on age, species, and mode of MPTP administration (Albanese *et al*., 1993; Ovadia *et al*., 1995; Taylor *et al*., 1997; Petzinger and Langston, 1998). In general, severely lesioned animals are less likely to recover than mildly lesioned animals, and intracarotid injection models are less likely to recover than systemic models (Taylor *et al*., 1997). For example, squirrel monkeys rendered severely parkinsonian by a series of six subcutaneous injections of MPTP over a 12-week period recover motor behavior within several months of their last injection (Petzinger *et al*., 2006). Figure 9.2 demonstrates differences in the time course of recovery of motor behavior depending on the degree of MPTP-lesioning. In contrast to systemic MPTP-lesioning, non-human primates administered MPTP via intracarotid injection tend not to recover motor behavior and may display stable parkinsonian features for months to years after lesioning. These differences in recovery between the various models likely reflect differences in the degree of midbrain dopaminergic cell loss. With systemic administration of MPTP, a proportion of SNpc dopaminergic neurons are spared (between 40% and 60%), whereas in the intracarotid paradigm almost no midbrain dopaminergic neurons remain ipsilateral to the site of injection. In fact, intracarotid lesioning can be so intense as to cause necrosis and stroke in the ipsilateral striatum (Emborg *et al*., 2006).

p0370 Studies investigating the mechanisms of recovery in these models have shown (i) alterations in dopamine biosynthesis (tyrosine hydroxylase) and metabolism (increased turnover); (ii) altered regulation of DAT expression and function; (iii) sprouting and branching of tyrosine hydroxylase fibers; (iv) alterations of other neurotransmitter systems including glutamate and serotonin; and (v) alterations of signal transduction pathways in both the



f0020 **FIGURE 9.2** Time course in motor recovery in the MPTP-lesioned non-human primate model. Squirrel monkeys ($N = 6$ per group) were administered saline, three or six injections of MPTP (2.0 mg/kg free-base, 2 weeks between injections). A CRS was administered to monitor parkinsonian features (Petzinger *et al.*, 2006). A score greater than 4 is considered the threshold for parkinsonian motor features. Animals receiving three injections of MPTP displayed mild transient parkinsonian features for only a few weeks while those receiving six injections showed moderate to severe parkinsonian features and showed full motor behavioral recovery 12 weeks after the last injection of MPTP. The open and gray bars represent the time frame of 6 and 3 injections of MPTP, respectively.

direct (D1 dopamine receptor) and indirect (D2 dopamine receptor) pathways (Chiueh, 1984a; Eidelberg *et al.*, 1986; Mori *et al.*, 1988; Nishi *et al.*, 1989; Rose *et al.*, 1989; Morgan *et al.*, 1991; Russ *et al.*, 1991; Cruz-Sanchez *et al.*, 1993; Frohna *et al.*, 1995; Bezard and Gross, 1998; Ho and Blum, 1998; Mitsumoto *et al.*, 1998; Rozas *et al.*, 1998; Rothblat *et al.*, 2001; Wade *et al.*, 2001; Jakowec *et al.*, 2004). Interestingly, the return of striatal dopamine is incomplete despite full motor recovery, although more pronounced return in the ventral striatum compared to the dorsal regions has been reported in several different MPTP-lesioned species (Elsworth *et al.*, 2000; Petzinger *et al.*, 2006). In the squirrel monkey we found that dopamine levels in tissue homogenate increased from 0.7% to 1.6% of baseline in the dorsal putamen at 6 weeks (when animals are moderately parkinsonian) and 9 months (when animals are fully recovered from motor impairment) (Petzinger *et al.*, 2006). However, in the ventral putamen dopamine levels at the same time points were 8.7% and 28.0% of baseline, respectively. One explanation may be that the ventral dopaminergic system is less sensitive to MPTP toxicity (Moratalla *et al.*, 1992). This raises the possibility that the ventral striatum, with a higher dopamine return than the dorsal, may play a role in behavioral recovery and one means may be through the diffusion of dopamine into the dorsal denervated regions (Schneider *et al.*, 1994). Even with insufficient total dopamine return, studies in the rodent models have shown that when dopamine loss is less than 80%, homeostatic mechanisms can lead to complete normalization of extracellular levels of dopamine; however, when dopamine depletion exceeds 80% there is only partial normalization (Robinson and Wishaw, 1988; Altar and Marien, 1989; Abercrombie *et al.*, 1990; Castaneda *et al.*, 1990). In our studies we also observed a dynamic change in protein expression in the caudate nucleus and putamen, where animals at 9 months after lesioning compared to animals at 6 weeks, showed increased levels of tyrosine hydroxylase and DAT protein that was more dramatic in the ventral than dorsal regions of the basal ganglia. Studies suggest that there are many pre- and postsynaptic molecular changes that occur as a consequence of dopamine denervation/dysfunction which may contribute to behavioral recovery and/or play a role in other phenomenology such as susceptibility to levodopa-induced dyskinesias. The fact that there are molecular and neurochemical changes that occur in a time course fashion in these models underscores the importance of considering the time

from lesioning as an important parameter in study design. Another cautionary point is that many of these molecular changes may take place in a time course fashion even in animals that do not display overt behavioral recovery. This raises the issue that studies should consider time since lesioning as a potential factor in influencing outcome measures when examining molecular and physiological changes.

ELECTROPHYSIOLOGICAL STUDIES OF BASAL GANGLIA FUNCTION IN THE NON-HUMAN PRIMATE MODEL OF PD

p0380 Electrophysiological studies in the normal and MPTP-lesioned non-human primate have provided valuable information regarding basal ganglia physiology and pathophysiology of PD and have led to the identification and testing of new therapeutic interventions. The utility of the MPTP-lesioned non-human primate is due in part to the following strengths: (i) the non-human primate shares similar basal ganglia structure and circuitry with humans and (ii) the MPTP-lesioned model demonstrates analogous pathological and clinical characteristics to idiopathic PD (Langston *et al*., 1983). For example, electrophysiological studies in the MPTP-lesioned non-human primate have provided evidence for increased activity in both the STN and globus pallidus and have suggested that abnormalities in the circuitry between nuclei of the basal ganglia underlie parkinsonian features. These findings supported the hypothesis that inactivation of the STN alleviates the parkinsonian features in the non-human primate and eventually led to the use of DBS surgery of the STN as a treatment for PD (Israel, 2007).

p0390 Electrophysiological studies in MPTP-lesioned non-human primate have also been valuable in examining alterations in the physiological properties of neurons and their molecular modulators within the basal ganglia and have identified new pharmacological targets. These studies have included the investigation of dopamine, glutamate, γ-aminobutyric acid (GABA), nicotine, and adenosine and their respective receptors, their influence on the physiological properties of medium spiny neurons of the striatum, and their effects on motor (including dyskinesia) and cognitive behavior (REF). For example, neurophysiological studies have implicated overactivity at corticostriatal synapses, due to altered glutamatergic neurotransmission, as one underlying mechanism for the development of motor impairment and levodopa related motor complications in PD. (Konitsiotis *et al*., 2000; Wichmann and DeLong, 2003; Soares *et al*., 2004). These findings have supported the clinical use of glutamatergic antagonists, such as amantadine, for the treatment of levodopa-induced dyskinesia in PD, and the concept that glutamatergic antagonists with receptor and anatomical specificity may provide a future therapeutic intervention for symptomatic treatment.

s0140 Electrophysiological studies in our labs, using the MPTP-lesioned squirrel monkey, have shown changes in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and GABA mediated synaptic neurotransmission that may account for excessive excitatory corticostriatal drive. For these studies, we administered MPTP in a series of six subcutaneous injections of 2.0 mg/kg (free-base) every 2 weeks for a total of 12 mg/kg. Whole brains were harvested at either 6 weeks (when animals are parkinsonian) or 9 months (when animals are motorically recovered) after the last injection of MPTP and striatal synaptic function was examined in coronal *in vitro* brain slices. We found that the input/output relationship was greater for AMPA-receptor-mediated synaptic currents at 6 weeks after MPTP-lesioning compared to saline control using whole cell voltage clamp. The relative strength of $GABA_A$-receptor-mediated synaptic inhibition versus AMPA-receptor-mediated synaptic excitation response was calculated as the I_{GABA-A} changes in the I_{GABA-A}/I_{AMPA} ratio. Interestingly, we also found a reduced I_{GABA-A}/I_{AMPA} ratio 6 weeks after MPTP. These GABAergic inhibition that we and others have observed may play an important role in facilitating the synchrony and oscillatory patterns of discharge found throughout the basal ganglia motor circuit in MPTP-treated akinetic primates (Raz *et al*., 1996; Raz *et al*., 2001; Goldberg *et al*., 2002). Analysis of animals 9 months after MPTP administration suggests that there is normalization of corticostriatal hyperactivity when animals demonstrate full behavioral recovery. Specifically we found the input/output ratio for AMPA-receptor-mediated synaptic responses and the I_{GABA-A}/I_{AMPA} ratio returned back to control levels (Figure 9.3). These observations are in agreement with the view that excessive glutamatergic corticostriatal synaptic function may be a contributing factor to the behavioral pathology of PD (Konitsiotis *et al*.,

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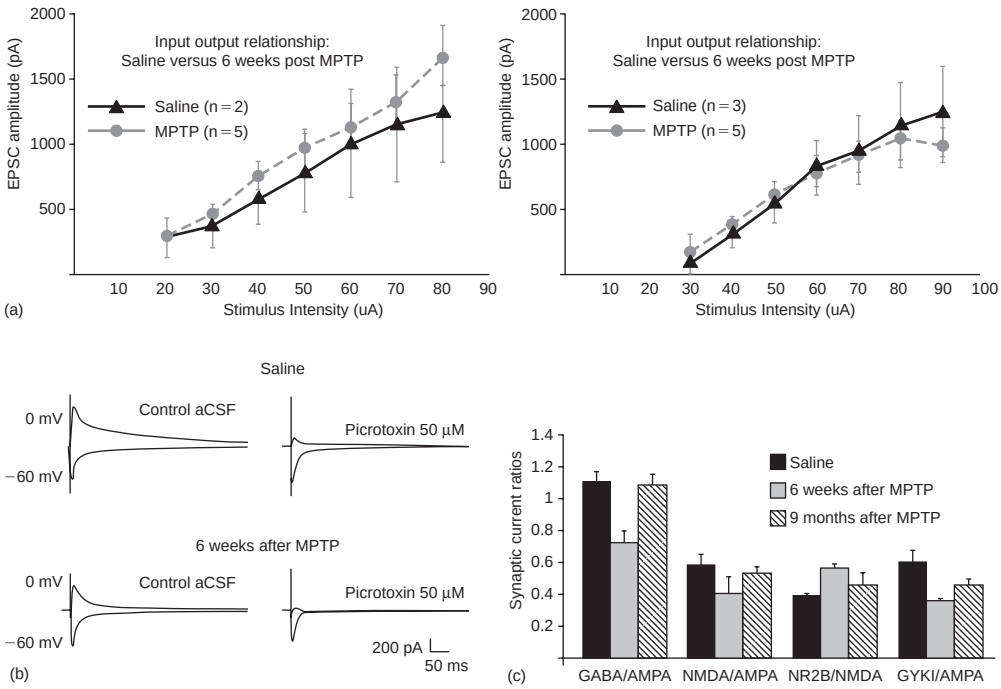

f0030 **FIGURE 9.3** Electrophysiological evidence for MPTP-induced changes in synaptic transmission. (a) Input (stimulus intensity applied to corpus callosum) – output (excitatory post-synaptic current or EPSC amplitude) relationships were determined for cortico-putamen synapses using whole cell voltage clamp with $GABA_A$ receptor blocked by picrotoxin. Note a greater tendency for larger amplitude EPSCs at 6 weeks post-MPTP (left panel), with a return to normal amplitude EPSCs by 9 months post-MPTP (right panel). (b) Example of synaptic currents recorded from a putamen neuron in response to corpus callosum stimulation before and after addition of picrotoxin. Synaptic currents were evoked in cells clamped at membrane potentials of –60 mV to maximize AMPA current activation and 0 mV to maximize $GABA_A$ current activation. (c) Ratios of synaptic currents illustrate a shift in synaptic function. At 6-week post-MPTP there was a reduction in the $GABA_A$/AMPA ratio, a reduction in the NMDA/AMPA ratio, an increase in the NR2B/total NMDA ratio, and a decrease in the GYKI 52466 sensitive/CNQX sensitive AMPA ratio. Interestingly, these trends returned to saline control levels by 9 months post-MPTP.

2000; Muriel *et al*., 2001). Future studies will exam whether changes in glutamatergic drive in fully recovered animals differentially impacts corticostriatal synapses in direct versus indirect basal ganglia pathways, as has been reported in the parkinsonian state (Wichmann and DeLong, 2003; Day *et al*., 2006). This could represent an additional consideration for therapeutic targeting.

p0410 The AMPA and N-methyl-D-aspartate (NMDA) receptors play a key role in determining the physiological properties of medium spiny neurons of the striatum. In the MPTP-lesioned non-human primate we observed changes in the pharmacological profile of AMPA and NMDA receptors, which are consistent with previously reported molecular studies in the dopamine denervated striatum (Betarbet *et al*., 2000; Betarbet *et al*., 2004; Nash *et al*., 2004; Hallett *et al*., 2005; Hurley *et al*., 2005). For example as shown in Figure 9.3, animals examined 6 weeks post-MPTP-lesioning displayed (i) a decrease in the I_{NMDA}/I_{AMPA} ratio; (ii) an alteration in the NMDA receptor subunit composition as indicated by increased sensitivity to the selective NMDA-NR2B antagonist CP-101606; and (iii) an alteration in AMPA-receptor-mediated synaptic responses, as indicated by changes in the sensitivity to the selective AMPA receptor antagonist, GYKI-52466 compared to saline control animals (Ruel

et al., 2002; Nash *et al*., 2004). Following behavioral recovery at 9 months post-MPTP-lesioning, there was a normalization of NMDA and AMPA receptor function toward saline control (Figure 9.3).

p0420 The glutamatergic corticostriatal and the dopaminergic nigrostriatal system are important mediators of synaptic plasticity, termed long-term depression (LTD) and long-term potentiation (LTP), within the basal ganglia (Centonze *et al*., 2001; Reynolds and Wickens, 2002; Mahon *et al*., 2004; Picconi *et al*., 2005). Electrophysiological studies in our lab, using saline control squirrel monkeys, have shown that the induction of long-term synaptic plasticity at corticostriatal synapses is region specific, with LTP being induced in more medial regions and LTD in more lateral regions. These findings agree with previous reports from the rodent model of PD (Partridge *et al*., 2000; Smith *et al*., 2001). Studies in the rat have shown a loss of synaptic plasticity after 6-OHDA administration, which we have observed in the MPTP-lesioned mouse model, 1–2 weeks after neurotoxicant exposure (Calabresi *et al*., 1992; Centonze *et al*., 1999; Kreitzer and Malenka, 2007). Presently, there is little known regarding alterations in synaptic plasticity immediately following MPTP-lesioning in the non-human primate.

p0430 Analysis of the expression of synaptic plasticity in the squirrel monkey 9 months after MPTP-lesioning has shown that LTD and LTP expression is evident. In the same animals used for analysis of glutamate neurotransmission above, we observed a dramatic and permanent decrease in dopamine release as measured by fast-scan cyclic voltammetry (Cragg, 2003) (Figure 9.4). This finding is in agreement with previous reports examining dopamine function in the squirrel monkey using HPLC (Petzinger *et al*., 2006). The expression of dopamine-dependent forms of LTP we observed in the dopamine-depleted squirrel monkey suggest that an adaptation may occur in the expression and/or sensitivity of both D1 and D2 dopamine receptors (Centonze *et al*, 2001; Reynolds and Wickens, 2002; Mahon *et al*., 2004; Picconi *et al*., 2005). Preliminary studies in our lab have shown that LTD expression at lateral cortico-putamen synapses from the 9-month MPTP-lesioned squirrel monkey is D2 dopamine receptor dependent, since this effect is blocked by the D2 dopamine receptor antagonist *l*-sulpiride. In addition, use of *l*-sulpiride results in the unexpected expression of LTP in lateral synapses (Figure 9.4). Our findings are consistent with the literature, where D1 and D2 dopamine receptors have been shown to play an important role in LTP and LTD, respectively (Calabresi *et al*., 1992; Centonze *et al*., 1999; Wang *et al*., 2006). Taken together, these data suggest behavioral recovery from MPTP exposure in the squirrel monkey may be due at least in part to compensatory increases in the sensitivity of dopamine receptors, which enables the normal and expected expression of long-term plasticity at corticostriatal synapses. The studies outlined above demonstrate how the MPTP-lesioned non-human primate model provides valuable insights regarding the role that neurotransmitters and their respective signaling pathways play in modulating the electrophysiological properties of basal ganglia neurons. These findings are serving to identify new therapeutic targets for treatment of PD.

OTHER NEUROTOXICANTS IN NON-HUMAN PRIMATES s0150

p0440 This chapter has focused on MPTP as the neurotoxicant to generate parkinsonism in the non-human primate since this is the most common regimen. Other neurotoxicants such as 6-hydroxydopamine, METH, proteasome inhibitors, and pesticides, while most often used in rodent models have had limited utility in non-human primates. The following sections highlight some features of these models.

6 Hydroxydopamine s0160

p0450 6-hydroxydopamine (6-OHDA or 2,4,5-trihydroxyphenylethylamine) is a specific catecholaminergic neurotoxin structurally analogous to both dopamine and noradrenalin. In addition to the rat, other species including the non-human primate (specifically the marmoset) have served as models for 6-OHDA lesioning (Annett *et al*., 1992; Eslamboli, 2005). Lesioning in non-human primates provides for the analysis of behaviors not observed in the rat, such as targeting and retrieval tasks of the arm and hand. This model, however, has not gained popularity for non-human primates because the toxin must be delivered directly in the vicinity of the dopamine cells by intracerebral injections. This is much more difficult method than administering MPTP systemically.

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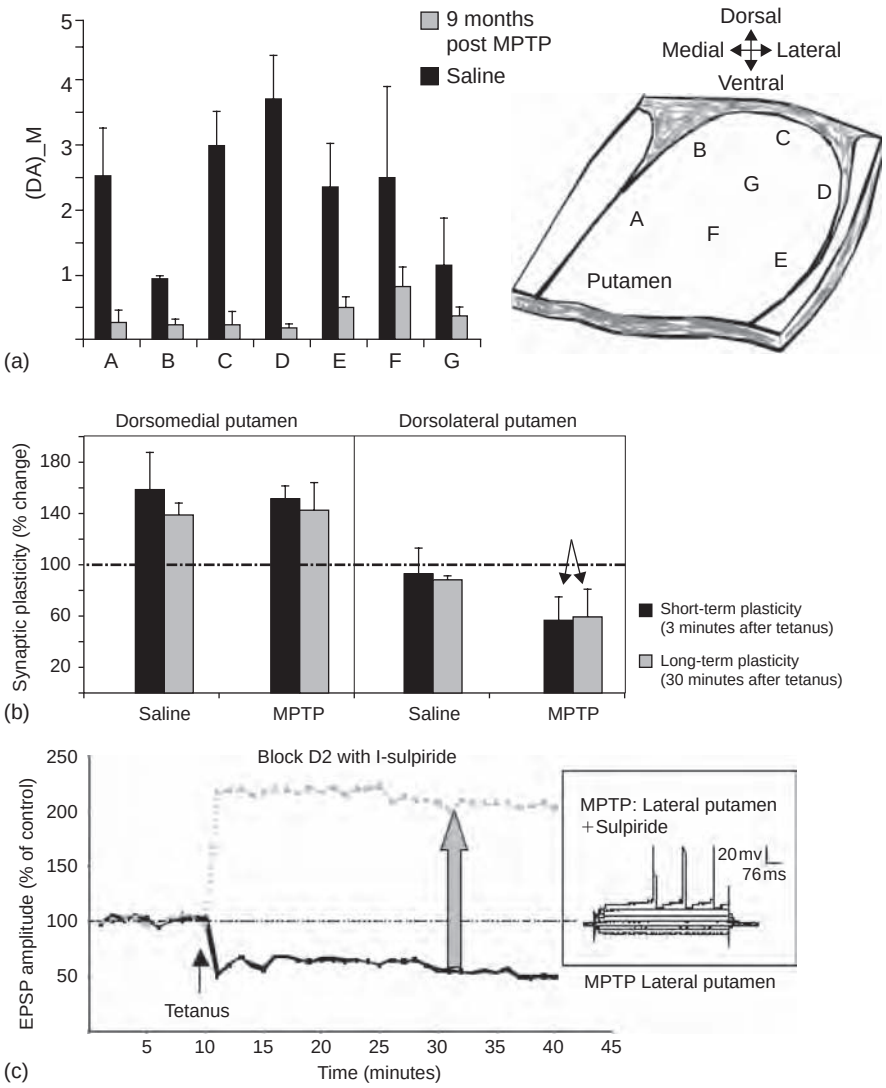

f0040 **FIGURE 9.4** Changes in dopamine release and synaptic plasticity in the MPTP-lesioned non-human primate. (a) Fast-scan cyclic voltammetry revealed regional differences in evoked dopamine release in the putamen that is markedly reduced even after 9 months post-MPTP when animals are motorically recovered. Letters in the graph correspond to putamen brain slice sites. (b) Comparison of short-term (3 min post-tetanus) and long-term (30 min post-tetanus) synaptic plasticity at corticostriatal synapses from saline and 9 post-MPTP-lesioning. Intracellular recording of EPSPs evoked at cortico-putamen synapses was used to monitor changes in strength induced by tetanic activation of the corpus callosum. Medial cortico-putamen synapses produced short- and long-term potentiation (LTP) in both groups. Lateral cortico-putamen synapses expressed short- and long-term depression (LTD) in saline and MPTP exposed monkeys, but the MPTP group tended toward greater LTD. (c) Example of LTD induced at lateral cortico-putamen synapses and tetanic activation of lateral cortico-putamen synapses in the presence of the D2 dopamine receptor antagonist *l*-sulpiride that enabled the expression of profound LTP. Inset shows the response of the putamen neuron recorded during the *l*-sulpiride experiment to current injection, which is typical of a medium spiny projection neuron.

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Methamphetamine

p0460 Amphetamine and its derivatives (including METH, *N*-methyl-beta-phenylisopropylamine) lead to long-lasting depletion of both dopamine and serotonin when administered to rodents and non-human primates including vervet, macaques, squirrel monkeys, and baboons (Ricaurte *et al*., 1980; Ricaurte *et al*., 1982; Villemagne *et al*., 1998; Davidson *et al*., 2001; Czoty *et al*, 2004). METH, one of the most potent of these derivatives, is typically administered in a series of small intramuscular or oral doses from 0.1 to 2 mg/kg and leads to dose-dependent terminal degeneration of dopaminergic neurons in the caudate nucleus and putamen, nucleus accumbens, and neocortex. Despite the severe depletion of striatal dopamine, the motor behavioral alterations seen in rodents and non-human primates tend to be transient and subtle.

In contrast to MPTP, which destroys nigrostriatal dopaminergic neurons and their terminals, METH administration spares axonal trunks and soma of SNpc and VTA neurons targeting terminals found within the caudate nucleus and putamen (Kim *et al*., 2000). Depending on the species and dosing regimen of METH, the effects of lesioning involves a spectrum from axonal degeneration to suppression of markers of nigrostriatal neuron integrity including tyrosine hydroxylase, DAT, and VMAT-2 proteins. The fact that these markers can be differentially affected by METH indicates that phenotypic suppression in dopaminergic neurons is a significant feature of METH exposure. In general, the effects of severe METH lesioning are long lasting. Interestingly, there is evidence of recovery of dopaminergic system depending on the METH regimen and species used (Harvey *et al*., 2000a). Studies employing PET-imaging in conjunction with histological analysis of markers of the dopaminergic system have demonstrated that re-establishment of the nigrostriatal system occurs which probably involves a combination of re-innervation (neuronal sprouting) and return of previously suppressed TH and DAT protein expression (Melega *et al*., 1997; Harvey *et al*., 2000b).

p0480 Similar to studies with MPTP, METH administration demonstrates the dynamic neuroplasticity of the nigrostriatal system and its ability to respond to neurotoxic injury. The administration of METH to adult animals has played an important role in testing the molecular and biochemical mechanisms underlying dopaminergic and serotonergic neuronal axonal degeneration especially the role of free radicals and glutamate neurotransmission. Understanding these mechanisms has led to the testing of different neuroprotective therapeutic modalities. An advantage of the METH model over MPTP is that the serotonergic and dopaminergic systems can be lesioned *in utero* during the early stages of the development of these neurotransmitter systems. Such studies have indicated that there is a tremendous degree of architectural rearrangement that occurs within the dopaminergic and serotonergic systems of injured animals as they develop. These changes may lead to altered behavior in the adult animal (Frost and Cadet, 2000). An finally, in light of the fact that METH and other substituted amphetamines (including methylenedioxymethamphetamine (MDMA) "ecstasy") are major drugs of abuse in our society, animal models have provided a means to understand the mechanisms of brain injury with these toxic compounds and to determine the long-lasting effects of these drugs including if humans who abuse METH are prone to develop parkinsonism (McCann *et al*., 1998; Guilarte, 2001; Paulus *et al*., 2002). p0470

Proteasome Inhibition

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p0490 It has been proposed that inhibition of the ubiquitin proteasome system (UPS) can lead to the inability to remove toxic protein moieties, accumulation of protein aggregates, neuronal dysfunction, and cell death (Petrucelli and Dawson, 2004; Tanaka *et al*., 2004). However, thus far modeling PD via a systemic administration of proteasome inhibitor has produced unreliable and irreproducible results (Kordower *et al*., 2006; Manning-Bog *et al*., 2006; Beal and Lang, 2006; Bove *et al*., 2006).

CONCLUSION

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p0500 To successfully translate findings in the laboratory to the clinical setting it is critical that novel experimental therapeutic approaches be evaluated in the non-human primate. Studies in normal animals can evaluate safety and tolerability issues, while studies in models of PD can test potential efficacy of pharmacological, surgical, and molecular approaches. Despite the fact that neurotoxicant models do not replicate all the pathological features seen in patients with PD, studies in non-human primate

models can help us better understand the human condition by providing a template to test hypotheses. For example, understanding why a therapeutic approach shown to be efficacious in rodent models but fails in patients with PD can be addressed in non-human primates and may reveal previously unknown or unrecognized features of the disease. The non-human primate serves as an important link bridging the phylogenetic continuum between rodents and *Homo sapiens*. In finding new therapeutic modalities for the treatment of neurological disorders such as PD many researchers feel that the non-human primate provides an essential model to validate findings in the preclinical phase.

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ACKNOWLEDGEMENTS

p0510 We would like to acknowledge the generous support of the Parkinson's Disease Foundation, Team Parkinson LA, and the Whittier PD Education Group, NIH RO1 NS44327-1 and RO1 AG21937, U.S. Army NETRP (Grant # W81XWH-04-1-0444). A special thanks to Friends of the USC Parkinson's Disease Research Group including George and Mary Lou Boone, and Walter and Susan Doniger. We thank our colleagues including Elizabeth Hogg, Pablo Arevalo, Jon VanLeeuen, Marta Vuckovic, Erlinda Kirkman, and Charles Meshul for their insightful discussions. Special thanks to Nicolaus, Pascal, and Dominique for their support.

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Movement Disorders, In Press

Title: Enhancing Neuroplasticity in the Basal Ganglia: The Role of Exercise in Parkinson's Disease

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Word Count: 1870

Running Title: Exercise in Parkinson's Disease

Abstract

Epidemiological and clinical trials have suggested that exercise is beneficial for patients with Parkinson's disease. However, the underlying mechanisms and potential for disease modification are currently unknown. This review presents current findings from our laboratories in PD patients and animal models. The data indicate that alterations in both dopaminergic and glutamatergic neurotransmission, induced by activity dependent (exercise) processes, may mitigate the cortically driven hyper-excitability in the basal ganglia normally observed in the parkinsonian state. These insights have potential to identify novel therapeutic treatments capable of reversing or delaying disease progression in Parkinson's Disease.

Keywords: dopamine, MPTP, animal models, treadmill, glutamate, electrophysiology, PET imaging.

Parkinson's disease (PD) is characterized as a progressive neurodegenerative disease with no known cure. The primary pathology of PD is loss of substantia nigra pars compacta neurons accompanied by loss of striatal dopamine. Exercise has been shown to be beneficial in PD, yet the question remains whether exercise leads to central nervous system (CNS) compensatory or neuroprotective changes with potential to alter the natural course of the disease. Studies have demonstrated that the adult brain is altered by experience including exercise. [1-4] This phenomenon termed "activity dependent neuroplasticity" is defined as modifications within the central nervous system (CNS), in response to physical activity that promotes a skill acquisition process.[5] As such (i) intensity; (ii) specificity; (iii) difficulty; and (iv) complexity of practice appear to be important parameters for driving neuroplasticity and a potential lasting effect on both brain and behavior. [6, 7] While the importance of these parameters have been primarily established in healthy brain and in brain injury secondary to stroke, this framework has more recently been adopted to study activity dependent neuroplasticity in neurodegenerative diseases, including Parkinson's Disease, and to examine its potential to modify disease progression (Table 1).[8-21]

Using a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-(MPTP)-lesioned mouse model of PD we have examined the effects of intensive treadmill exercise on activity dependent neuroplasticity within the striatum. Our studies have focused on exercise-induced changes in dopaminergic and glutamatergic neurotransmission. Interactions between these systems are important for normal basal ganglia function. Both dopaminergic neurons from the substantia nigra as well as glutamatergic afferents from the cerebral cortex and thalamus synapse in close proximity on medium spiny neurons (MSN) of the striatum, dictating the electrophysiological properties of these cells.[22, 23] There is compelling data that the loss of nigral dopaminergic neurons is responsible for an increase in glutamatergic corticostriatal drive at the level of the MSNs, contributing to the motor deficits in PD. [24-27] One possible mechanism by which exercise may drive activity-dependent neuroplasticity in PD may be through mitigating corticostriatal hyperactivity (i.e., hyperexcitability), by modulating dopaminergic signaling and/or diminishing glutamatergic neurotransmission.

Changes in Dopaminergic Neurotransmission with Exercise

Our MPTP model consisted of administration of four intraperitonal injections of 20 mg/kg (free-base) at 2-hr intervals for a total administration of 80 mg/kg, which leads to 60-70% of nigrostriatal dopaminergic neuronal death. Five days post-lesioning, when cell death is complete, mice were subjected to exercise on a motorized treadmill for 30 days (5 days/week). Task specific benefits were observed as improvements in both running velocity and endurance. Improvement was also observed on a motor task that was designed to assess balance.[20] These benefits were accompanied by increased dopamine availability, revealed as an increase in stimulus-evoked release and a decrease in dopamine decay as measured by fast-scan cyclic voltammetry. Interestingly this exercise effect of dopamine release was most pronounced within the dorsolateral striatum. Use dependent forms of neuroplasticity may explain this regional specificity in an exercise-induced effect. Additionally we observed an increase in expression of dopamine D2 receptor mRNA and down regulation of the dopamine transporter (DAT) protein within the striatum, changes that are consistent with increased dopaminergic signaling .[19] A primary role of DAT is to clear dopamine from the extracellular space. Down-regulation of DAT protein leads to increased synaptic dopamine availability for dopamine receptor binding.[28] The binding of dopamine to both the D1 and D2 receptors are required in the normal brain to elicit a motor response. After basal ganglia injury, however, this synergy is lost and dopamine binding to either D1 or D2 may elicit a motor response.[29] In addition, dopamine binding to the D2 receptor alone may elicit a robust response that may be attributed to its heightened sensitivity after lesioning.[30] Thus, an exercise-induced increase in D2 receptor expression coupled with an increase in the synaptic availability of dopamine may be sufficient to elicit increased dopaminergic neurotransmission and improved motor function. Preliminary Positron Emission Tomography (PET) imaging studies in our lab using 18F-Fallypride, a benzamide ligand with high affinity for the D2 receptor, have demonstrated an exercise-induced increase in binding affinity within the striatum, confirming our D2 receptor findings. Interestingly we observed no exercise-induced changes in either the total level of striatal dopamine, as measured by HPLC in tissue homogenates, or the number of dopaminergic substantia nigra neurons, measured by immunohistochemistry. These findings suggest that high intensity exercise leads to compensatory changes in dopamine handling and neurotransmission.[20]

Changes in Glutamatergic Neurotransmission and Exercise

Studies in our laboratory also suggest that exercise-induced neuroplasticity of the glutamatergic system may diminish corticostriatal hyperexcitability and underlie the motor improvement observed in our exercised mice. Specifically, using immuno-electron microscopy we have observed that treadmill exercise reversed the MPTP-induced increase level of *presynaptic* glutamate immunolabeling within striatal terminals, suggesting that exercise reduced the amount of glutamate available for release.[19] In addition, new studies in our lab demonstrate that treadmill exercise modulates *postsynaptic* AMPA receptor subunit expression through an increase in both GluR2 and phosphorylation of GluR2 at serine 880. [31] The potential process by which these changes may lead to decreased glutamatergic hyperexcitability could involve a general reduction in glutamatergic neurotransmission and synaptic strength (i.e., long-term depression). We have been interested in examining exercise-induced changes in the AMPA-type glutamate receptor (AMPAR), since it is responsible for the majority of fast excitatory neurotransmission in the CNS and it mediates activity dependent processes that alter synaptic strength.[32, 33] Located on the post-synaptic MSN, the AMPAR is an ionotropic channel that converts the chemical signal of presynaptically released glutamate into a post-synaptic electrical signal through the mobilization of cations such as Na^{2+} and Ca^{2+}.[34] The AMPAR is a heteromeric tetramer consisting of four subunits GluR1-4; the most abundant in the striatum are GluR1 and GluR2.[32, 35] Alterations in GluR2 expression and phosphorylation have been associated with diminished synaptic strength (i.e. long-term depression).[32, 36-38] Increased expression of the GluR2 subunit within the tetrameric complex of the AMPAR, as seen in our exercised mice, creates an additional positive charge within the channel pore, which impedes cation flow, lowers calcium conductance and thus diminishes synaptic strength.[34, 39] Another means of regulating AMPAR transmission occurs via trafficking and removal of the AMPAR from the membrane. This may be regulated through phosphorylation of AMPAR subunits, including GluR2. Specifically, phosphorylation of serine 880 on the GluR2 subunit leads to internalization of the entire receptor and decreased synaptic strength (i.e. long-term depression).[33, 40] Studies in our laboratory reveal that treadmill exercise increases the phosphorylation state of GluR2 at serine 880 in MPTP-lesioned mice. Additional electrophysiological studies indicate that exercise-induced changes in the expression of GluR2 subunit lead to decreased excitability in the MSNs, demonstrated by reduced EPSCs generated by cortical stimulation. They have also shown reduced polyamine sensitivity and loss of rectification in AMPA receptor conductance at depolarized membrane potentials of MSNs. These findings provide further evidence that changes in GluR2 expression are the basis for the exercise-induced reduction in the EPSCs of MSNs.[34, 39, 41] Finally, we have observed exercise-mediated attenuation in cortico-striatal hyper-excitability in cerebral perfusion studies in rats with bilateral striatal lesions. Collectively our data in both mouse and rat models of basal ganglia injury indicate that exercise training attenuates the over activation in basal ganglia-cortical circuits.

In summary, these findings suggests that alterations in both dopaminergic and glutamatergic neurotransmission through activity dependent processes modulates cortical hyper-excitability of the basal ganglia. Modulation of cortical hyper-excitability may be what underlies exercise-induced behavioral improvement. An important next step is to translate these findings to humans, and to investigate whether high intensity exercise has similar benefits in PD.

Activity-Dependent Neuroplasticity and Parkinson' Disease

Since our studies in animal models suggested that high intensity is a characteristic of exercise that may be specifically important in promoting activity-dependent neuroplasticity, we designed a study to the use of Body-weight supported treadmill training (BWSTT) to drive intensity of practice in individuals with PD. BWSTT involves the use of an overhead harness that allows exercise intensity to be safely escalated by increasing treadmill velocity. Thus subjects are able to walk at higher gait speeds than they are able to obtain over-ground. They also experience high repetition of stepping, are actively engaged in the training, and have the sensory experience of normal gait kinematics. Patients with PD, no more than 3 years from initial diagnosis were asked to exercise at high intensity, 3 times per week for 8 weeks using body-weight BWSTT. Outcomes consisted of measures of motor performance, including gait kinematics, sit-to-stand, and stair climbing. Unique to this human trial, and directly related to our animal finding, was the inclusion of measures of cortical excitability using transcranial magnetic stimulation (TMS). TMS is a noninvasive method of stimulating the brain and provides a tool for assessment of excitability of the corticospinal motor system. Single TMS pulses are applied over the motor cortex while recording surface electromyography (EMG) responses over a contralateral target muscle. If the target muscle is pre-activated (contracted), the TMS pulse induces a characteristic transient period of EMG silence called the cortical silent period (CSP). Importantly for this study,

single pulse TMS studies have shown systematic abnormalities of CSP and other corticoexcitability measures in individuals with PD. In general, these abnormalities reflect cortical hyper-excitability in PD compared to non-PD control subjects.[42, 43] As CSP represents inhibitory influences on cortical excitability, higher excitability would be evident as a *shortened* CSP duration. In fact, shortened CSP durations are among the most consistent and widely reproduced TMS finding amongst PD patients.[44] Further, symptomatic treatment of PD with surgical or pharmacological interventions is associated with lengthening of the CSP towards levels seen in control subjects. [45, 46] Thus CSP duration could underlie symptomatic improvement, such as improved motor performance. Thus, not only is TMS an excellent tool to measure CSP duration and to examine possible exercise-induced changes in PD, but more importantly TMS may be used to support the existence of CNS changes in response to different exercise parameters including intensity. After 24 sessions of BWSTT subjects demonstrated improved walking performance including increased gait velocity, stride length, step length, and hip and ankle joint excursion, and improved weight distribution during sit-to-stand. More importantly, these subjects also showed reversal of cortical hyper-excitability indicated by increased CSP. In fact every subject undergoing BWSTT showed exercise-induced lengthening of CSP. To our knowledge this was the first demonstration of exercise-induced cortical changes in the brain in individuals with PD.

Future Directions

We have shown that exercise may influence activity-dependent processes in the basal ganglia through alterations in dopaminergic and glutamatergic neurotransmission. In addition, we demonstrate that exercise-induced behavioral benefits may be due in part to changes in cortical hyper-excitability normally observed in the dopamine depleted state, as in PD. While we have demonstrated the potential impact of BWSTT on the human condition, a critical next step is to determine whether exercise induces or is associated with a disease modifying effect in PD. The implications for our understanding of the impact of exercise in PD are broad. Not only is there potential to develop new insights into mechanisms of neuroplasticity and motor recovery in PD, but the study of exercise may lead to the development of novel therapeutics, perhaps even non-pharmacological approaches to delay or reverse disease progression in PD.

Acknowledgments: All authors have provided substantial contributions to the data presented in this manuscript. This work was supported by the Parkinson's Alliance, Team Parkinson LA, the Whittier Parkinson's Disease Education Group, National Institute of Neurological Disorders and Stroke Grant R01 NS44327-1, and the United States Army Neurotoxin Exposure Treatment Research Program Grant W81XWH-04-1-0444. A special thanks to friends of the University of Southern California Parkinson's Disease Research Group, including George and Mary Lou Boone and Walter and Susan Doniger. We thank Dr. Wendy Gilmore for her insightful discussions and editing of the manuscript.

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Table 1: Practice Variables important for evoking activity-dependent neuroplasticity- Examples in Brain injury (PD, Stroke, Spinal Cord Injury)

Practice Variable	Animal Study	Human Study
Intensity	Petzinger et al., 2007; Tillerson et al., 2001	Liepert , 2006; Liepert et al., 2000;
Specificity	Fisher et al., 2004; De Leon et al., 1999 Tillakaratne., 2002	Forrester et al., 2006; Dobkin et al., 2004
Difficulty	Friel & Nudo, 1998	Wittenberg et al., 2003; Johansen-Berg et al., 2002
Complexity	Jones et al., 1999	Winstein et al., 1997

Version Final

The effects of exercise on basal ganglia function in Parkinson's disease and its animal models.

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Abstract

Recent studies in animal models of Parkinson's disease, as well as epidemiological reports, have ignited an interest in the potential application of exercise as a treatment modality not only for symptomatic benefit but also for its capacity to modify disease progression. This important goal is based on the premise that the adult brain, even in a state of injury or a neurodegenerative disorder, displays tremendous neuroplasticity that is influenced by experience. The objective of this chapter is to present findings that may help us to gather evidence that exercise, a form of experience-dependent neuroplasticity, influences basal ganglia circuitry and motor control, and to examine findings that may elucidate the underlying mechanisms responsible for this phenomenon. Examining the impact of exercise in both the normal and injured brain in animal models will guide us to develop testable hypotheses that can be translated to studies in patients with Parkinson's disease and to develop improved treatments.

Keywords: MPTP, 6-hydroxydopamine, dopamine, glutamate, treadmill, running wheel, neuroplasticity, repair, motor behavior, clinical trials.

Introduction

The beneficial effects of physical therapy and general forms of exercise in individuals with Parkinson's disease (PD) have been reported in numerous studies [1-8]. Epidemiological studies have also revealed that increase in physical activity levels, particularly strenuous exercise, throughout life has been associated with a lower risk of developing neurodegenerative disorders such as PD [9-11]. These studies have served as a catalyst to further determine whether exercise may represent a critical component of therapeutic intervention in PD, and more importantly, to investigate whether exercise leads to central nervous system (CNS) compensatory or neuroprotective changes with potential to alter the natural course of disease. Over the past few decades, studies have demonstrated that the brain is altered by experience including exercise. This phenomenon termed experience-dependent neuroplasticity is defined as modifications within the CNS in response to physical activity that promotes a skill acquisition process. As such (i) intensity; (ii) specificity; and (iii) complexity of practice appear to be important parameters for driving neuroplasticity with potential lasting effects on both brain and behavior. While the importance of these parameters have been primarily established in both the healthy and brain injury secondary to stroke, this framework has been more recently adopted to study experience dependent neuroplasticity and particularly the effects of exercise on neurodegenerative diseases, such as PD, and to examine its potential to modify disease progression. The purpose of this chapter will be to highlight findings from exercise studies in normal brain and toxin-induce animal models and in individuals with PD, focusing on aspects important to basal ganglia circuitry and motor control.

The traditional approach to physical therapy intervention for individuals with PD has been focused on teaching patients compensatory strategies to bypass symptoms associated with basal ganglia pathology as well as to prevent secondary strength and mobility impairments that may arise from disuse. The use of external cues and cognitive strategies has been considered the practitioner's main training options. Thus individuals with PD are instructed to consciously process movement information such as thinking about swinging the arms or taking large steps. In addition, the physical demands of many of the exercise protocols have been at low to moderate levels in intensity. The goal of traditional therapy has been largely to help people maintain what motor capability they have for as long as possible and to help them adjust as their functional level inevitably declines. This traditional approach to physical therapy intervention i.e., teaching compensation for motor deficits, stems from the assumption that in the case of a neurodegenerative process such as that seen in PD, there is no potential for neurological recovery.

This assumption has been challenged by (i) the recognition that the adult brain has capacity for recovery from injury and importantly (ii) the demonstration that exercise can promote brain plasticity and increase neuroprotective and neuroregenerative processes. In addition, in the last few decades there has been accumulating evidence that brain injury and progressive degeneration may support an environment by which experience can more readily facilitate neuroplasticity through molecular, eletrophysioglogical, or structural events that could enhance functional outcome [12; 13].

The Effects of Exercise on Normal Brain.

Studies in normal (non-injured) animals have been extremely valuable in elucidating the neurobiological basis for the beneficial effects of exercise. These studies support that exercise effects on the brain may be related to up-regulated mechanisms of neuroprotection, neuroplasticity, neurogenesis, and/or angiogenesis. While we classify these exercise effects as being categorically distinct, there is tremendous overlap with respect to their potential role in modulating behavior and function. The following sections examine some of these features.

Neurotrophic Factors

Animal studies examining effects of exercise on the brain have shown that physical activity, primarily running can increase neurotrophic factors, including brain derived neurotrophic factor (BDNF), glia-derived neurotrophic factor (GDNF), neuronal growth factor (NGF), fibroblast growth factor (FGF), and insulin-like growth factor 1 (IGF-1). Exercise-induced up-regulation of neurotrophic factors have been documented in a number of brain regions involved in learning, memory, mental processing, mood, and motor control and include the hippocampus, cerebellum, cerebral cortex, and striatum. [14-17]. Neurotrophic factors in the CNS are expressed at different levels in a number of various cell types including neurons, microglia and astrocytes. The precise mechanism that may trigger neurotrophic expression with exercise is not fully elucidated. Metabolic demand, neurotransmitter release, and other released factors may serve as intrinsic local agents of neurotrophic factor activation. In addition, the peripheral immune system may contribute to exercise-induced alterations in neurotrophic factor levels and may complement CNS derived sources.

BDNF is one major neurotrophic factor closely linked to exercise. Increased expression of BDNF in response to exercise may be an important factor in exercise-derived benefits [17-20]. BDNF is a neurotrophin widely distributed throughout different regions of the brain. It provides both neurotrophic and neuroprotective support to many subpopulations of neurons throughout both development and adulthood. BDNF is a key mediator of synaptic efficacy and experience-dependent neuroplasticity and is associated with both learning and memory processes within the hippocampus. Exercise modulates the induction of BDNF in a time-dependent manner within the hippocampus and cerebral cortex contributing to brain plasticity and functional maintenance [21]. BDNF mRNA and its protein appear quickly within days in rodents undergoing voluntary running wheel exercise and induced levels are sustained even several weeks after completion of an exercise regimen. Exercise also primes the molecular memory for BDNF induction where a brief second session of exercise intervention can more rapidly re-induce BDNF protein expression. The induction of the major receptors for BDNF through exercise, including the tyrosine kinases TrkB and p75, are also important for experience-dependent neuroplasticity since the balance between these two receptors can dictate the preferences for long-term potentiation (LTP) or long-term depression (LTD) within the hippocampus and striatum [22; 23]. The molecular details underlying the beneficial effects of exercise mediated through BNDF are not yet fully elucidated. However, it is believed that neurotrophic factors may promote neurogenesis, angiogenesis, and synaptogenesis at dendritic sites (local effects) or cell bodies of neurons and glia (global effects) through (i) activation of kinases, including the mitogen activated kinase (MAPK) cascades, (ii) induction of local protein translation within dendrites thus modifying synaptic morphology and synaptic strength, (iii) induction of gene expression at the level of the cell nucleus, or (iv) the regulation of presynaptic neurotransmitter release. These mechanisms play a key role in lowering the threshold for potentiating activation of processes involved in neuroplasticity and repair.

Neurogenesis and Exercise

Physical activity may have neuroregenerative and/or neuroprotective influences by stimulating neurogenesis, the birth and development of new cells. Within the normal adult brain specific anatomical regions displaying robust neurogenesis has been well documented including within the dentate gyrus of the hippocampus, the subventricular zone, and the olfactory bulb [24-27]. Newborn neurons are thought to replace dying or defective neurons directly through neurogenesis within these anatomical sites or are translocated to specific regions as a result of movement from other brain regions via routes such as the rostral-caudal migratory pathway [28; 29]. Experience is one means thought to enhance neurogenesis. For example, the initial identification of enhanced neurogenesis within the hippocampal dentate gyrus was reported in rats housed under conditions of environmental enrichment with objects to promote social interactions, reduce stress, and provide voluntary exercise with running wheels [30; 31]. Interestingly, when the individual components used in environmental enrichment were separated it was revealed that the running wheel itself was predominantly associated with enhancing neurogenesis [32; 33] and that neurogenesis in the context of voluntary exercise, in contrast to enriched housing, has potentially distinct pathways [34]. Subsequent studies using a variety of techniques have shown that normal rodents subjected to treadmill running results in potentiating neurogenesis within the dentate gyrus of the hippocampus due to the influences of blood flow [35], neurotrophic factors [36], and glutamate neurotransmission [37]. Neurogenesis within the basal ganglia circuitry, either within the substantia nigra where midbrain dopaminergic neurons reside or within the striatum has not yet been

demonstrated in the context of exercise. However, two studies in the 6-OHDA lesioned rat subjected to environmental enrichment have demonstrated enhanced neurogenesis within the striatum based on doublecortin immunoreactivity [38] and within the substantia nigra based on labeling for BrdU-incorporation [39]. The precise role of neurogenesis within the brain is far from complete. Newborn neurons could replace dying neurons, expand the neuronal population, or act as protection from insult or injury. Interestingly, the fact that behavioral benefits of environmental enrichment may not necessarily require hippocampal neurogenesis [40] raises questions regarding the role of neurogenesis in this region as well as its role at sites of injury. Since voluntary running wheel exercise has been shown to elevate neurotrophic expression within the hippocampus it is thought that factors such as BDNF, IGF-1, and other neurotrophic factors have a major effect on promoting neurogenesis as well as synaptogenesis and cell survival [16; 41; 42; 43].

Blood Flow and Angiogenesis

Physical activity, including running and endurance training, has been shown to increase blood flow, angiogenesis, and to alter factors involved in maintaining the integrity of the blood brain barrier. Exercise induces angiogenesis and blood flow within the cerebellar and motor cortices [44-46; 47). These cerebrovascular changes that occur in response to exercise are likely due to changes in metabolic demand of underlying neural circuits . While the precise mechanisms involved in exercise-induced increase of blood flow and angiogenesis are not clearly elucidated, some basic understanding is beginning to emerge. Potentially important factors include angiopoetin and vascular epithelial growth factor (VEGF). VEGF, initially termed vascular permeability factor, is constitutively expressed to maintain vessel integrity, and its elevation in expression in conjunction with its receptor results in increased angiogenesis and increased permeability of the blood brain barrier. Angiopoetin also increases local angiogenesis but unlike VEGF does not have as pronounced an effect on blood brain barrier permeability. Mechanisms underlying VEGF elevation by exercise are not clear, but one possible process may involve changes in oxygen demand where a state of hypoxia may directly trigger VEGF expression. Another source of increased VEGF expression is thorough IGF-1 where both local CNS and peripheral sources of IGF-1 can elevate transcription factors such as hypoxia inducible factor 1 (HIF-1) leading to induction of VEGF. The role of VEGF in disrupting the blood brain barrier may be important for recruiting factors from the periphery such as activated macrophages, cytokines, or other molecules which may also participate in local exercise-induced angiogenesis. Physical activity has also been shown to increase astrocytic and epidermal cell proliferation in both the motor cortex and striatum, which may reflect alterations in the blood brain barrier, and contribute both angiogenesis and passage of factors from the peripheral to central nervous system [16; 36; 48]. Interestingly, different forms of exercise may mediate various degrees of angiogenesis. For example, endurance training creates a significant local metabolic demand leading to increased angiogenesis while skilled motor training might not [44; 49].

Synaptogenesis and Dendritic Morphology

Changes in dendritic spine density and morphology induced by physical exercise through skilled motor training have been well documented in several regions of the normal adult rat brain including the motor cortex, hippocampus, and cerebellum. For example, long-term voluntary running increases the density of dendritic spines in the entorhinal cortex and hippocampus [50], as well as granule cells of the dentate gyrus in adult rats [51]. A study involving rats trained to navigate successfully through a complex obstacle course showed that after five to ten days of motor skill training there was a significant increase in the synaptic connectivity as measured by synapse-to-neuron ratio within the motor cortex [52]. In addition, similar training of adult rats induced an increase in dendritic arborization of stellate interneurons within the cerebellar cortex [53]. Finally, another study that used adult rats undergoing a motor skill learning task for thirty days, showed a significant increase in the number of synaptic connections in the cerebellum between parallel-fiber and climbing-fiber inputs to Purkinje neurons [54].

While exercise related mechanisms in the normal brain have been well documented, the effects of exercise as an intervention on the injured brain, including the role of neurotrophic factors, neurogenesis, synaptogenesis, and angiogenesis on experience-dependent neuroplasticity are only recently being explored. In addition, in the context of a neurodegenerative disorder, like PD, it is unclear to what extent these exercise related processes observed in the normal brain may modify disease progression.

Exercise in Animal Models of Parkinson's disease

Parkinson's disease is characterized by loss of nigrostriatal dopaminergic neurons and the depletion of striatal dopamine and these features are replicated in both the 1-methyl-4-phenyl-1,2,3,6-tetrahydopyridine (MPTP) and 6-hydroxydopamine (6-OHDA) lesioned animal models. Consequently, the focus of exercise studies in PD has been to examine for neuroprotective or neurorestorative effects on the dopaminergic system and its down-stream targets within basal ganglia circuitry. Exercise studies examining for neuroprotection or neurorestoration are important since major goals in PD research are to prevent dopaminergic neuronal loss, to promote survival of remaining neurons, and to enhance compensatory mechanisms important for basal ganglia function and motor performance. Table 1 summarizes published animal studies examining the effects of exercise on the basal ganglia and its circuitry in normal and dopamine depleted rodent models. In the next sections we highlight some of the findings from these studies.

Exercise and Neuroprotection

An important goal of exercise studies in animal models of PD (using neurotoxicant lesioning) is to determine whether exercise attenuates the degree of injury to midbrain dopaminergic neurons or limits subsequent secondary neurodegeneration. The administration of either 6-OHDA or MPTP to rodents leads to midbrain dopaminergic cell death and depletion of striatal dopamine, and provides two important animal models to study the effects of exercise on basal ganglia injury [55; 56]. Studies examining the effects of motor enrichment or exercise exposure prior to 6-OHDA or MPTP administration show attenuation of motor deficits and neuroprotection of dopaminergic neurons. Exercise-induced neuroprotection is demonstrated with respect to: (i) the neuronal integrity of dopamine production and handling through gene and protein expression of tyrosine hydroxylase (TH), dopamine transporter (DAT) and vesicular transporter type-2 (VMAT-2); (ii) the survivability of midbrain dopaminergic cells through comparative cell counts and/or; (iii) the maintenance of dopaminergic neuronal integrity through analysis of striatal dopamine levels and its metabolites. Specifically, exercise initiated one day prior to 6-OHDA or MPTP lesioning and continued for one week post-injury showed improved motor behavior, attenuation of striatal dopamine depletion, and a reduction in the loss of the dopaminergic markers TH, DAT, and VMAT-2 when measured at completion of the exercise regimen [57]. The application of intense motor-training (forced-use) on the impaired limb in the

unilateral 6-OHDA-lesioned rat also provides protection of dopamine integrity and motor behavior when used either pre-lesioning or within the first week post-lesioning [58; 59]. However, starting forced-use at time points greater than seven days post-lesioning showed no benefit indicating that the phases of neuroprotection and lack of efficacy may be quite distinct. One hypothesis accounting for neuroprotection in these exercise studies is through the elevation of neurotrophic factors such as BDNF, GDNF, or IGF-1, which could provide protection by activating downstream signaling cascades including second messenger systems and protein kinases that may enhance neuronal function [60]. An alternative hypothesis accounting for exercise-induced neuroprotection from both 6-OHDA and MPTP is a reduction in the bioavailability of the neurotoxin. For example, dopaminergic neurons may be protected due to exercise-induced alterations in the expression of DAT or VMAT-2, transporters important for neurotoxin uptake either at the dopaminergic neuronal terminal or vesicular uptake from the cell cytoplasm, respectively [61-63]. Alterations in the blood brain barrier or xenobiotic detoxification in the liver may also affect brain levels of neurotoxin. The time course of exercise intervention relative to the neurotoxicant administration/exposure is also important since both MPTP and 6-OHDA have prolonged periods of toxin-induced cell death ranging from three to twenty-eight days for MPTP and 6-OHDA, respectively, and an exercise-induced suppression of DAT and/or VMAT-2 during this time frame could result in reduced bioavailability and subsequent cell death [64; 65]. Two studies using environmental enrichment that included an exercise component in the form of a voluntary running wheel showed protection from MPTP-lesioning was likely due to suppression of DAT and VMAT-2 protein expression in midbrain dopaminergic neurons [66; 67]. Studies from O'Dell and colleagues using pre-lesioning voluntary exercise with a forced-exercise component post-lesioning for four weeks showed improvement in motor performance but without protection of 6-OHDA-induced injury of midbrain dopaminergic neurons and suggested alternative mechanisms for exercise benefits including compensatory changes in remaining dopaminergic neurons and/or increased function of other striatal pathways [68].

Exercise and Neurorestoration

After injury, there exists an opportunity to enhance neuroplasticity through exercise, including skilled or voluntary running. Using the MPTP-lesioned mouse model of PD, we have examined the effects of intensive treadmill exercise starting five days after the last injection of MPTP when neurotoxicant-induced cell death is complete. Our studies have focused on exercise-induced compensatory changes of the dopaminergic and glutamatergic systems, two systems important for normal basal ganglia function and motor control.

Changes in Dopaminergic Neurotransmission with Exercise

Intensive treadmill exercise in the post-injury state leads to improved motor performance and dopaminergic signaling within the basal ganglia. Specifically intensive treadmill training initiated five days after MPTP administration (when cell death is complete) and continued for thirty days (five days per week, one hour per day) leads to task specific benefits in both running velocity and endurance as well as improved balance using an accelerating rotarod [69]. The MPTP model for these studies consisted of the administration of four intraperitoneal injections of 20 mg/kg (free-base) at two-hour intervals for a total administration of 80 mg/kg, which results in a 60-70% destruction of nigrostriatal dopaminergic neurons and a greater that 95% depletion of striatal dopamine levels. Similar to what is seen in patients with PD, this lesioning regimen spares a subset of surviving neurons, which may subsequently act as a template for experience-dependent neuroplasticity [70; 65]. In our exercise paradigm, behavioral benefits from treadmill exercise were accompanied by an increase in evoked dopamine release within the striatum and decrease in extracellular decay as measured using fast-scan cyclic voltammetry. Enhanced dopamine release was most significant in the dorsolateral region of the striatum, a region involved in motor control [71; 69]. Additionally, treadmill exercise led to down regulation of the DAT (measuring both mRNA transcript and protein) within the striatum. The elevated evoked release of striatal dopamine from surviving nigrostriatal dopaminergic terminals, in conjunction with reduced levels of DAT (the primary route for pre-synaptic removal of dopamine), leads to an increased synaptic occupancy of dopamine and hence increased dopamine neurotransmission [72; 73]. Analysis of the pattern of expression of the dopamine receptors also showed an exercise-induced elevation of dopamine D2 receptor (DA-D2R), but no change in the expression of the dopamine D1 receptor (DA-D1R) in striatal MSNs. Studies using ^{18}F-Fallypride with PET imaging have also shown an exercise-induced increase in the DA-D2R. ^{18}F-Fallypride is a high affinity D2/D3 receptor radioligand that can be used to detect changes in DA-D2R expression within the basal ganglia after exercise. Interestingly there were no exercise-induced changes in either the total level of striatal dopamine, as measure by HPLC homogenates, or the number of substantia nigra neurons, supporting the role of high intensity exercise in modulating compensatory changes in dopamine handling and neurotransmission in surviving dopaminergic neurons. In support of the exercise-induced changes of the DA-D2R, a similar effect has been observed using in vitro receptor autoradiography in the striatum of normal rats after chronic exercise [74]. DA-D2R activation is important for normal function of the basal ganglia, establishing LTD, and motor learning and may provide a key factor for compensation after injury [75; 76]. Some of the effects of dopamine neurotransmission may be thorough glutamate. When DA-D2R are activated or blocked, glutamate release decreases or increases, respectively [77-79], thus indicating that alterations in the level of DA-R expression and/or dopamine levels can have profound effects on nearby glutamate neurotransmission. Taken together these findings suggest that increased dopamine neurotransmission through the DA-D2R pathway may represent one important underlying mechanism for experience-dependent neuroplasticity, and the functional benefits of exercise.

Changes in Glutamatergic Neurotransmission with Exercise

In addition to the dopaminergic system, studies from our lab and others support that the beneficial effects of exercise may be due to alterations in glutamatergic neurotransmission within the basal ganglia and its circuitry. Glutamatergic projections either from the corticostriatal or thalamostriatal pathways onto medium spiny neurons (MSNs), play a major role in mediating basal ganglia function and motor control [80-83]. There is compelling evidence that the loss of nigral dopaminergic neurons, followed by the depletion of striatal dopamine, is responsible for an increase in glutamatergic corticostriatal drive leading to hyperexcitability at the level of the MSNs, contributing to the motor deficits in PD [84-87]. A possible mechanism by which exercise may lead to beneficial effects in PD is through the attenuation of corticostriatal hyperexcitability. Studies in our laboratory suggest that exercise-induced neuroplasticity of the glutamatergic system may diminish corticostriatal hyperexcitability and underlie the motor improvement observed in our exercised mice. Specifically, using immuno-electron microscopy we observed that thirty days of treadmill exercise initiated five days after MPTP lesioning, reversed the MPTP-induced increase in the level of *presynaptic* glutamate-immunolabeling within striatal terminals, suggesting that exercise reduced the amount of glutamate available for release [88]. In addition, recent studies demonstrate that

treadmill exercise also modulates *postsynaptic* glutamate receptors, including the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA-R) subtype, in the MPTP-lesioned mouse model. The AMPA-R subtype of glutamate receptors is responsible for the majority of fast excitatory neurotransmission in the CNS and regulates activation of N-methyl-D-aspartate receptors (NMDA-Rs) [89; 90]. AMPA-R are located on the post-synaptic MSN and act as ionotropic channels that convert the chemical signal of presynaptically released glutamate into a post-synaptic electrical signal through the mobilization of cations such as Na^+ and Ca^{2+} [91]. Pathological elevation of AMPA-R function in PD could act to depolarize striatal MSNs into a region of instability, through the recruitment of NMDA-R and voltage-dependent Ca^{2+} channels, which could lead to oscillatory bursts of activity. Indeed, modeling studies have shown that changing AMPA/NMDA receptor ratios by itself changes the oscillatory behavior of ventral striatal MSNs [92]. We found that the AMPA/NMDA receptor ratio increased in MPTP mice and that high-intensity treadmill exercise reduced the AMPA/NMDA receptor ratio back towards the level seen in saline mice (unpublished observation). These data not only add support to exercise as a modality for restoring normal corticostriatal drive, but also for reducing pathological oscillations in the basal ganglia seen with dopamine depletion. In addition to the exercise-induced changes in glutamate receptor expression, we also observed changes in AMPA-R subunit composition after treadmill running. The electrophysiological properties of AMPA-R channels are influenced by its subunit composition. The AMPA-R is a heteromeric tetramer consisting of four subunits GluR1 through GluR4 with the most abundant in the striatum being subunits GluR1 and GluR2 [93; 89]. Studies in our laboratory reveal that treadmill exercise leads to increased expression of the GluR2 subunit in MPTP-lesioned mice. AMPA-R channels with predominant GluR2 subunit composition have been shown to reduce the unitary conductance of AMPA receptors and to reduce the Ca^{2+} permeability of AMPA-R thus serving as an important mechanism for diminishing postsynaptic hyperexcitability due to dopamine depletion [94; 95]. A fundamentally important correlate to our findings using molecular techniques is to validate AMPA-R expression with changes in the electrophysiological properties of striatal MSNs. Using organotypic slice cultures we have examined the effects of treadmill exercise on excess excitation and/or alterations in the frequency of discharge in striatal MSNs in MPTP-lesioned mice. Stimulation of corticostriatal afferents in these *in vitro* brain slice preparations produced large AMPA-R mediated synaptic events (in the form of EPSCs; excitatory post-synaptic currents) in MPTP-lesioned mice that were reduced back to normal after exercise (see the comparison of input/output relationship in Figure 1). Our molecular findings in conjunction with our electrophysiological results suggest that the exercise-mediated reduction in the size of corticostriatal synaptic events could be due, at least in part, to an increase in the expression and thus influence of GluR2 subunits [91; 96; 97].

In summary, these findings suggest that alterations in both dopaminergic and glutamatergic neurotransmission through experience-dependent processes modulate cortical hyperexcitability of the basal ganglia. Thus modulation of corticostriatal hyperexcitability may underlie exercise-induced behavioral improvement. An important next step is to translate these findings to humans, and to investigate whether high intensity exercise has similar benefits in patients with PD.

Exercise-Induced Neuroplasticity in Brain Injury

An exciting advance in neuroscience over the last two decades has been the recognition that the capacity for recovery from injury in the human adult brain is far greater than previously thought. It is now recognized that the brain has the ability to reorganize and undergo neuroplastic changes after disease or injury and that this phenomenon can be facilitated through experience-dependent processes including forced use, complex skills training and exercise. Most of our understanding of this experience dependent neuroplasticity is derived from studies of brain injury related to stroke and spinal cord injury. Lesion models (rodent and nonhuman primate) in the cortex have permitted investigation of neuroplasticity by studying the effect of forced active use on body segment(s) impaired by brain injury. In addition, basic science research has provided substantial evidence that functional locomotor recovery occurs in animal models of stroke and spinal cord injury when intense, locomotor training is employed.

Noninvasive imaging and stimulation techniques now offer evidence that experience-dependent reorganization occurs in the human brain in response to exercise, locomotor training and motor skill learning. This experience-dependent neuroplasticity has been shown to play a major role in the recovery of function after stroke. An important example of this is preliminary work using functional magnetic resonance imaging (fMRI) before and after treadmill training with body weight support [98; 99]. Use of body-weight-supported treadmill training (BWSTT) involves an overhead harness that can ease the transition into ambulation. Because patients are protected from falling, BWSTT allows them to safely train at greater walking speeds. The intensive, task-specific practice of BWSTT promotes neural recovery rather than compensation as evidenced by significant improvements in velocity of locomotion, motor control of the hemiparetic limbs and alterations in fMRI activation patterns [98; 99].

Physical Therapy and Exercise in Parkinson's disease

Historically, the goal of exercise programs for PD has not been to examine whether we can induce neuroplasticity since the general belief has been that the potential for experience-dependent neuroplasticity would be limited in a neurodegenerative process. Rather the goal has been to maintain the highest possible level of function as people inevitably decline. Exercise studies in mild to moderate states of PD have focused on avoiding secondary impairments from disuse such as weakness and limitations in mobility. Some approaches have been to teach the patient with PD to rely on external cues (visual, auditory) to compensate for impaired internal generation of behavior normally derived from activity in cortico-basal ganglia loops [100].

Over the last fifty years there have been numerous studies demonstrating the beneficial effects of exercise in individuals with PD [1-8; 101-103]. An analysis of exercise studies in PD over this period shows that overall the physical demand of the exercise protocols for the most part were low to moderate in intensity. In addition, the activities within the studies could be grouped into six categories: (i) passive range of motion (ROM) and stretching; (ii) active ROM; (iii) balance activities; (iv) gait; (v) resistance training; and (vi) practice of functional activities and transitional movements (i.e., sit-to-stand). These studies have shown that exercise is an important adjunct to pharmacological treatment and helps walking ability and activities of daily living, as well as neurological symptoms, such as slowness, stiffness and balance dysfunction [104; 105].

More recently, a number of studies examining the effects of treadmill training have shown that individuals with PD can benefit from treadmill exercise in which gait behavior is driven more automatically. Improved motor performance has been reported and treadmill speeds have gradually increased from studies in which subjects trained at self-selected velocities for comfort to speeds above over-ground walking velocity. For example, Pohl et al. (2003) examined the short-term effects of unsupported, speed-dependent treadmill training on patients with early PD [106]. Significant, immediate differences were observed following a single treadmill training intervention versus conventional gait therapy in both walking speed and stride length [106]. In two separate randomized, controlled trials Miyai et al. (2000, 2002) [107; 108] compared immediate and long-term effects of two interventions for individuals with PD. The interventions were BWSTT and a standard physical therapy regimen involving general conditioning, range of motion, and gait training exercises. After twelve treatment sessions, the BWSTT group exhibited significantly greater changes in disease severity ratings as determined by the Unified Parkinson's Disease Rating Scale (UPDRS), whereas the conventional therapy group showed negligible differences. Furthermore, both velocity and cadence over a distance of ten meters improved post-treatment in the BWSTT group, which persisted even after a four-week period of no training. A similar report from Toole et al. (2005) using BWSTT demonstrated functional benefits after six weeks that was sustained 4 weeks later [109]. While greater than in previous studies, the intensity of training was relatively modest. For example in Miyai et al. (2000) patients were allowed to exercise at self-selected treadmill speeds [107].

A recent trend has demonstrated that patients with PD can participate in and benefit from more intensive exercise. In a study by Hirsch et al. (2003) subjects with PD participated in ten weeks (three times a week) of high-intensity lower extremity resistance *and* balance training and were assessed before, immediately after training, and four weeks later. Muscle strength and balance increased substantially and this effect persisted for at least four weeks [8]. Additionally, Dibble et al. (2006) examined changes in quadriceps muscle volume, muscle force production, and mobility as a result of a twelve-week high-force eccentric resistance training program in PD. Eccentric resistance training led to muscle hypertrophy, improved strength, and mobility in persons with PD [110].

While exercise interventions are gradually becoming more intense an important next step is to examine if, similar to what has been seen in the animal models described above, intensive exercise in PD leads to neuroplastic changes and disease modification. Human epidemiological studies support this hypothesis by demonstrating a neuroprotective role of strenuous exercise [9; 111]. Borrowing from our treadmill studies in MPTP-lesioned mice, we are currently using intensive exercise with BWSTT to explore the potential for brain changes and concomitant behavioral improvement in PD. In order to examine the potential disease-modifying role of exercise, our studies have focused on earlier stages of disease where the chances of detecting significant exercise effects may be optimized.

We recently completed a randomized, control trial to examine the effects of high-intensity exercise using BWSTT on functional performance in people with early stage PD, (Hoehn and Yahr stage one or two) relative to exercise at low (physical therapy) and no intensity. An important part of the study was to determine whether improved performance was accompanied by changes in the brain (neuroplasticity) as measured through transcranial magnetic stimulation (TMS). TMS is a noninvasive neuro-imaging technique that assesses the responsiveness (excitability) of the corticomotor system, Subjects in the two exercise groups completed twenty-four exercise sessions over eight weeks, while subjects in the zero-intensity group completed six education classes over eight weeks. Subjects in the high-intensity exercise group worked on average at a metabolic equivalent level (MET) of 4.3 with a range between 2.5 and 13.3 METS and speeds ranging from 8.0 to 12.8 km/h (5.0–8.0 mph). Proper gait kinematics for stance and swing (upright posture, extending and flexing the hip, knee, and ankle and coordinating limb movements to achieve symmetric limb cadence and equal step length) were maintained during the treadmill training. Subjects in the PT group participated in individualized exercise sessions, which included active and passive range of motion, strengthening, gait and balance activities. In this study, we found that people with PD participating in high-intensity BWSTT improved in kinematic measures of gait performance (i.e., step length, stride length, hip extension excursion), and lower-extremity symmetry of ground reaction forces during a sit-to-stand task. This improvement was not observed consistently in the other groups. In addition using TMS, we observed lengthening of maximal cortical silent period (CSP) duration in all subjects in the high-intensity exercise group. Changes in CSP duration were not observed in subjects in the low-intensity and no exercise groups. Among TMS studies examining corticomotor excitability in patients with PD, CSP durations are among the most consistent abnormalities reported, with generally shorter duration reflective of increased corticomotor excitability and associated with greater parkinsonian symptoms. Similar to the observed effects of high-intensity exercise, CSP durations are increased in PD patients after taking levodopa, apomorphine and pergolide; drugs known to provide effective symptomatic relief of motor symptoms. Findings from our study demonstrate the capability of individuals with PD to engage in intensive exercise and suggest a potential role of intensity of exercise in driving experience-dependent neuroplasticity and functional improvement in people with PD and warrants further investigation.

Conclusions

While the benefits of exercise and physical therapy in PD is recognized, only recently has there been an interest to examine the potential effects of exercise on brain function, and more specifically on disease modification. To this end, studies have utilized more challenging and complex interventions designed to retrain and re-educate normal motor function. Animal models provide an opportunity to test specific hypothesis regarding the mechanisms by which exercise leads to experience-dependent neuroplasticity. In addition, neurotoxin-induced animal models, including rodents and potentially nonhuman primates, as well as some of the recently developed transgenic mouse models of PD, can begin to explore yet uninvestigated fundamentally important aspects of exercise including the role of the central and peripheral immune systems, the effects of exercise on non-motor features including affective behavior (anxiety and depression), learning, and other cortical functions such as attention and executive function. In light of the potential application of progenitor and stem cell, as well as various virus-based vector construct approaches as treatments for disorders such as PD, successful survival and integration of these agents can be influenced by the target environment including brains undergoing exercise [112]. Current and future studies in both healthy and PD animal models provide the evidence for exercise induced neuroplasticity and thus have ignited the interest and supported the rationale for this new exercise direction in humans.

Acknowledgements

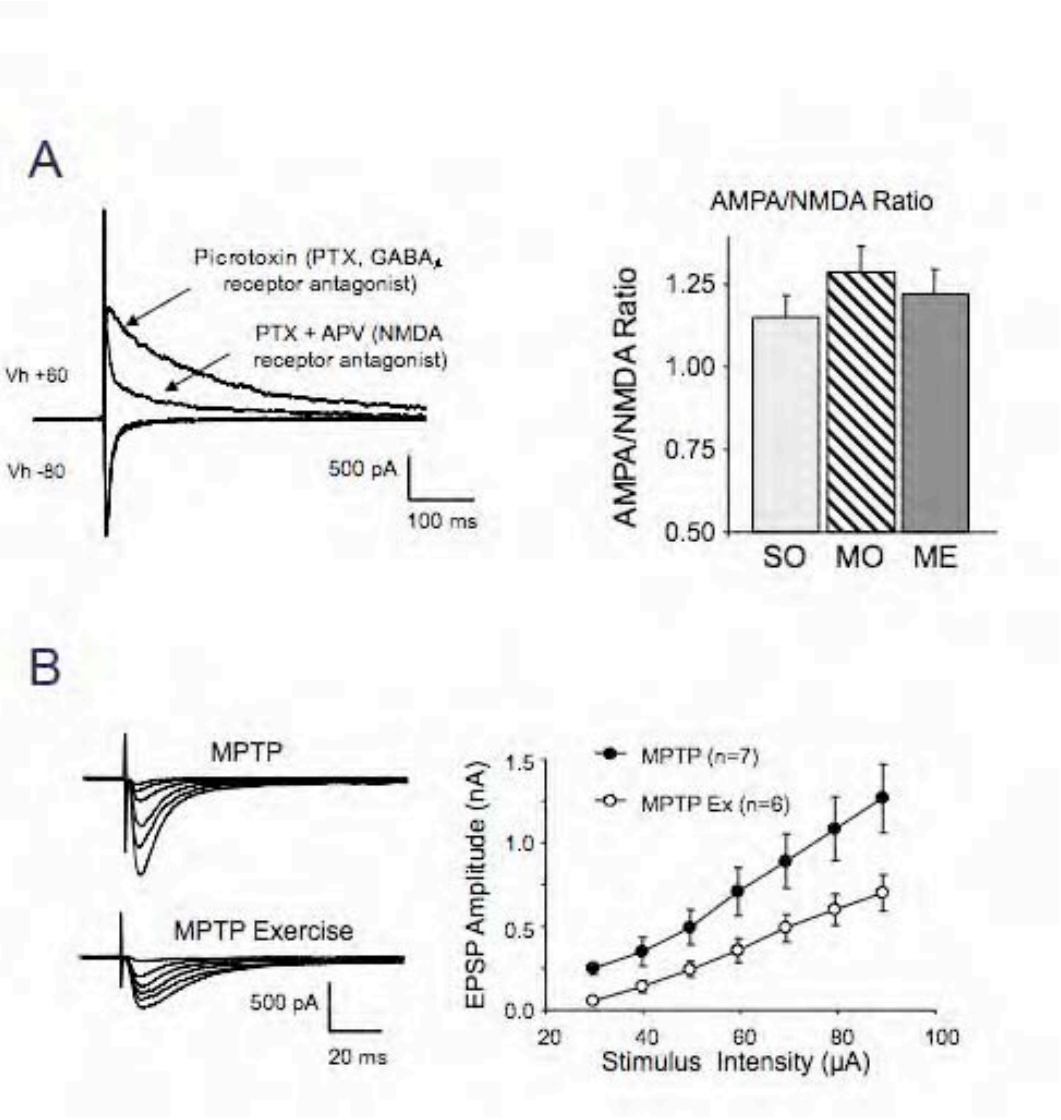
We would like to thank our colleagues for their helpful discussions and contributions including Daniel Holschneider, Marta Vuckovic, Breyda Ortega, Jon VanLeeuwen, Wendy Gilmore, Brett Lund, Ruth Wood, and Michael Zigmond. Studies discussed in this review were supported from the US Army NETRP, NIH, Team Parkinson, The Parkinson Alliance, Whittier PD Support Group, and Kinetics Foundation. This chapter is dedicated to the memory of Dr. George Boone whose vision and support inspired many of the studies from our labs.

Figure and Table Legends

Table 1: Listing of published studies examining the effects of exercise or skilled training on basla ganglia circuitry and motor control. These studies were carried in rodents without dopamine lesioning or with subject lesioned with 6-OHDA or MPTP. This Table serves as a synopsis of the variations in rodent models used and outcome measures evaluating either neuroprotection or neurorestoration. Abbreviations: AMPH, amphetamine; Apo, apomorphine; BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; CORT, corticosterone; CPu, caudate putamen; DA, dopamine; DA-D1R; dopamine D1 receptor; DA-D2R, dopamine D2 receptor; DAT, dopamine transporter; DBH, dopamine beta-hydroxylase; EE, environment enrichment; GDNF, glia-derived neurotrophic factor; GLU, glutamate; HPLC high performance liquid chromatography; 5-HT, serotonin; HVA, homovallinic acid; LE, Long Evans; MFB, medial forebrain bundle; NE, norepinephrine; NPY, neuropeptide Y; PPE, preproenkephalin; PPT, preprotachykinin; SD, Sprague-Dawley rats; SE, standard environment; SN, substantia nigra; TH-ir, tyrosine hydroxylase immunoreactivity; VTA, ventral tegmental area.

Authors	Species	Lesioning Regimen	Exercise Regimen	Outcome Measurements	Results/Notes/Conclusions
MacRae et al 1987a (113)	SD Rats; 3 or 18 months of age;	None	Treadmill running, screen 2 weeks at 5-10 min/day; Controls: 5 min/day at 5-10 m/min for 2 days/wk; Trained group: 12 weeks ramping to 20 m/min for 1 hr, 5 days/wk.	DA, DOPAC, HVA by HPLC DA-D2R (spiperone) binding	Showed 27% enhancement of citrate synthase activity in older exercised rats. Older runners had higher striatal DOPAC levels. No changes in DA levels. Higher HVA/DA ratio with older runners. Elevated D2-R binding in older runners.
MacRae et al 1987b (74)	Rats SD 3 mo. Old	None	Same as MacRae 1987a except used 6 months of treadmill. Compared young control vs young runner.	DA, DOPAC, HVA by HPLC DA-D2R (spiperone) binding	Exercise increases D2-R binding and stabilizes DA metabolism. The beneficial effects of exercise may be enhanced in aging.
Dishman et al 1997 (114)	Rats S-D 1 mo. old	None	Activity Wheel (motorized)	Escape latency from foot-shock. DA, 5HT, and their metabolites by HPLC.	Exercised rats had 34% reduction in latency to escape foot-shock. Change in NE and 5HT suggest exercise reduced anxiety and depression. No changes in DA.
Bland et al 1999 (116)	Rats Long-Evans	None Casting	Spontaneous movement in cylinder.	Glutamate by microdialysis.	Spontaneous movement increased extracellular levels of Glu. Casted animals showed reduced Glu on contralateral side. Use-dependent arborization may involve Glu.
Meeusen et al 1997 (115)	Rats Wistar	None	Exercise 5 days/wk for 6 weeks from 19 m/min for 30 min to 26 m/min for 80 min.	Microdialysis after 1 hr exercise in trained and control rats.	Trained rats increased extracellular GLU levels in striatum, reduced basal activity of striatum, levels of DA and GLU reduced. Propose a functional link between GLU and DA.
Humm et al 1999 (117)	Rats Long Evans	Lesion of sensorimotor cortex (FL-SMC)	Some lesioned rats the affected limb was casted and some received MK-801. Sham controls were included.	Behavior with forelimb placing and cylinder tests. Microdialysis in CPu for glutamate. TH-ir of SN to sample cell number.	Blocking glutamate spared SN neuron loss. And blocked use-dependent exaggeration of cortical injury. Forelimb placing ability recovered by blocking glutamate but not landing from rearing position.
Tillerson et al 2001 (58)	Rats Long Evans	6-OHDA MFB	Forced use of impaired limb by casting for 1-7, 3-9, or 7-13 days after lesioning.	Behavioral testing for limb akinesia, wall exploration, limb placement, Apo rotation. DA and metabolites, DAT, TH, VMAT2 protein.	Early cast showed no difference in limb asymmetry compared to control in a range of measures. Reduced or no benefit to casting at later time points. Early casting protected DA levels from depletion. Partial benefit seen in day 3-9 casting.
Tumer et al 2001 (118)	Rats Fisher S-D old	None	Motorized treadmill, gradual increase in speed and duration over 2 weeks reaching 1 hour/day running.	Analysis of mRNA expression for TH, NPY, DBH. Mobility shift assay for AP-1 and CREB.	Increased TH mRNA in old rats but not young after exercise in SN. Increased TH mRNA in VTA and LC of young rats.
Tillerson et al 2002 (119)	Rats Fisher Mice C57BL/6 (retired breeders)	6-OHDA to MFB; MPTP 2x15, 12 hours apart	Treadmill 1 day prior to lesion at 15 m/min for 10 min. Baseline analysis 2 hours after lesion. Rats run day 1-9, 15 m/min for 2x15 min. Mice on treadmill 1 day before lesion at 5 m/min and then from day of last lesion to 9 days at 5 m/min for 2x5 min.	Behavioral testing for limb asymmetry, limb placement, stride length, grid-walking (length and faults), HPLC of DA, WIB for TH, DAT, VMAT2.	Treadmill running resulted in recovery from limb asymmetry and akinesia, protected from behavioral deficits seen in MPTP lesion mice, resulted in normalization of DA levels and sparing of TH, DAT, and VMAT2 depletion. Suggest exercise-induced sprouting. Neuroprotective effect leaving more surviving cells. No assessment of degree of lesioning.
Tillerson et al 2002 (120)	Rats Long Evans	6-OHDA MFB mild, severe	Casted unaffected forelimb days 1-7 after lesioning for forced-use followed by period of non-use.	Behavioral testing limb asymmetry, akinesia, placing. Apo-rotation, HPLC of DA, and WIB for TH, DAT, VMAT2.	Casting the unimpaired forelimb in first 7 days resulted in enhanced injury.
Bezard et al. 2003 (66)	Mice C57BL/6	MPTP 4 x 20mg	Weaned at 21 days than in EE for 2 months. EE large cage, toys, running wheel. SE standard cage	Cocaine behavior in activity cages. MPP+ levels, D1-R, D2-R, Enk, BDNF, TrkB, DAT mRNA	EE mice less responsive to cocaine, altered c-fos pattern and reduced novel locomotor reactivity, fewer SNpc neurons, resistant to MPTP, with same levels MPP+ in EE and SE mice. DAT binding and mRNA reduced in EE. BDNF increased but trkA/B unchanged. D1-R, D2-R, PPE, PPT mRNA unchanged.
Cohen et al 2003 (59)	Rats Long Evans and SD	6-OHDA MFB and limb casting	None; casting of one limb for disuse. Fore limb was cast for 7 days prior to lesion in the ipsilateral side.	Behavior in forelimb asymmetry and akinesia. Apo-induced rotation. DA by HPLC. Striatal GDNF levels.	Casted rats prior to lesioning showed reduced forelimb asymmetry and akinesia with lesioning, attenuated both contralateral turning and loss of DA. Elevated GDNF in striatum corresponding to cast limb that returned to baseline.
Dobrossy et al. 2003 (112)	Rats S-D	6-OHDA 10 weeks posttransplant	EE with visual stimulus and more intense training than deprived groups	Apomorphine induced rotation. Paw reaching and skinner box. Striatal TH-ir to assess graft survival	Rat in EE showed increased graft survival with improved behavior and reduced rotations.
Verara-Aragon et al 2003 (122)	Rats Long Evans	6-OHDA MFB	Skilled reaching task taught to rats fro 14 days 3 weeks after lesioning.	Behavioral testing included skilled reaching, cylinder test, APO-induced rotation. TH-ir	Results suggest improved reaching due to development of compensatory movements ipsilateral to the lesion through training.
Fisher et al 2004 (88)	Mice C57BL/6 10 wks old	MPTP 4 x 20 mg/kg, free-base	Treadmill exercise started 4 days after MPTP for 30 days (6 days/wk). Ramping 10- 25 m/min, 1 hr/day	Analysis after 30 days. Behavior (velocity and duration), TH and DAT striatal protein, D1 and D2 striatal mRNA. Immuno-EM for glutamate	Enhanced velocity and endurance in MPTP+E. Exercise suppressed DAT and TH protein, elevated D2 but not D1 mRNA, and normalized immunogold labeling in striatum.
Mabandla et al 2004 (123)	Rats Long Evans	6-OHDA MFB	Running wheels 7 days before lesioning and than 14 days after lesioning.	Apo-induced rotation	Exercised rats did not rotate suggesting protection.
Faherty et al 2005 (67)	Mice C57BL/6	MPTP 4x20 mg	Housed in EE, exercise, or standard cages. Running wheels provided but not monitored.	WIB for TH for cell counting. PCR for BDNF, GDNF, DAT, VMAT2. MPP+ in subset of mice.	EE in adult mice provides protection from MPTP. Elevated striatal BDNF in EE. No change in GDNF in striatum but elevated in SN. Reduced DAT and VMAT2 in EE and exercise.
Jadavji et al, 2006 (128)	Rats Long Evans	6-OHDA MFB	EE and SE before lesioning. Skilled reaching and skilled walking.	Behavior test skilled walking and reaching, open field, forelimb asymmetry, Apo-induced rotation TH-ir, CORT	More SN TH-ir neuron in EE rats. Improved behavior and lower CORT levels in EE rats.
Howells et al. 2005 (124)	Rats Long Evans	6-OHDA MFB	Voluntary running wheel in home cage. Groups runners, runners+stress, sedentary. Running started 1 week before lesion, continued for 3 weeks, 2 weeks sedentary.	Behavior with apomorphine induced contralateral rotation. Sample SNpc TH-ir neurons.	Running was neuroprotective. Stress reduced the benefits of running.
Li et al, 2005 (48)	Rats S-D	None	Motorized treadmill, 15 m/min, 30 min/day, 6 weeks	Astrocyte proliferation by GFAP-ir in dorsolateral striatum and cortex.	Elevated astrocyte activation in exercise groups that persisted after exercise completion.
Poulton and Muir 2005 (125)	Rats Long Evans Age 9-10 months	6-OHDA MFB	All 2 week runway training. Treadmill 3x20 min 2 weeks prior lesioning at 0 or 13 m/min. Treadmill started 24 hrs or 7 days after lesioning for 6 days/week for 30 day period at 13 m/min.	Extent of DA depletion evaluated 2 wks after lesion. Behavioral testing at 3 and 6 weeks post-lesion for forelimb akinesia, ladder walking (stride, speed, errors). Ground reaction force, DA.	Treadmill caused attenuation of DA loss. Treadmill training did not ameliorate behavioral deficits. No return of symmetrical pre-surgical gait. Used ratio of L and R hemispheres for DA by HPLC. Suggest that studies that starting intervention too early are in fact providing protection from lesion event.
Steiner et al. 2006 (39)	Rats SD	6-OHDA striatum	EE with rotarod and SE	AMPH-induced rotation. BrdU labeling, ICC for TH-ir.	EE rats had increased numbers of BrdU labeled TH-ir cells and glia in the SN. EE rats improved rotation.
Al-Jarrah et al. 2007 (126)	Mice C57BL/6 10-12 wks old	MPTP + probenacid 2 x 25 mg/kg/ wk for 5 wks	Motorized treadmill 18 m/min, 40 min/day, 5 d/wk, for 4 wks.	Behavior open field with AMPH challenge. ECG. VO2, VCO2, HR. Citrate synthase. HPLC of DA, DOPAC. TH-ir CPu, SN	In lesioned mice, exercise led to reduced heart rate, restoration of R-wave amplitude, decline in VO2, increased AMPH-induced locomotor activity. Slight elevation of TH. No change in DA or DOPAC.
Anstrom et al, 2007 (129)	Rats SD	6-OHDA MFB	Focused sensorimotor training by vibrissae-elicited forelimb placing before/after 6-OHDA	Behavior placing test, spontaneous limb use, TH-ir in SN and striatum.	Intervention showed behavioral benefits and protection from DA depletion and cell loss.
O'Dell et al. 2007 (68)	Rats S-D	6-OHDA striatum	Voluntary or forced running or running wheel starting 2.5 weeks before lesioning and continuing for additional 4 weeks.	Behavioral stepping test, cylinder test, elevated grid. DAT autoradiography. TH-ir in midbrain to count SNpc neurons.	Running resulted in recovery of motor deficits without recovery of DAT or TH-ir neurons.
Yoon et al. 2007 (127)	Rats S-D	6-OHDA striatum	Treadmill exercise was started 1 day after lesioning at 2-3 m/min for 14 days.	Apomorphine rotation. Striatal TH-ir. SN cell TH-ir counts by sampling.	Treadmill exercise lesioned rat had 33% less rotation, 30% more TH-ir cell, and 10% greater striatal TH-ir than 6-OHDA non-exercise rats.
Petzinger et al, 2007 (69)	Mice C57BL/6	MPTP 4x20 mg/kg (free base)	10 week old mice, treadmill exercise started 5 days after MPTP. Treadmill exercise for 30 days (5 days/wk). Ramping 10- 25 m/min, 1 hr/day	Analysis after 30 days of exercise. Behavior (velocity and duration), TH and DAT striatal protein, midbrain DA cell counts, HPLC analysis of striatal DA DA release by voltammetry	Motor behavioral recovery not accompanied by increased DA or change in midbrain DA cell number. Increased evoked release of DA in dorsolateral striatum.
Urakawa et al. 2007 (38)	Rats Wistar	6-OHDA striatum or SN	EE with running wheels and SE	Beam walking, cylinder test, BrdU-labeling, TH-ir,	EE rats showed improved behavior, no cell loss protection with EE, more migrating cells in EE, different response in SN compared to striatal injury.

Figure 1. Electrophysiological measures of corticostriatal excitation. Panel A: *AMPA receptor participation is reduced with exercise in the MPTP lesioned mouse*. The voltage clamp traces on the left illustrate experimental approach for measuring AMPA and NMDA receptor-mediated synaptic responses. The bar graph on the right indicates MPTP-lesioning increased the AMPA/NMDA ratio, while exercise in the MPTP-lesioned mouse reduced the AMPA/NMDA ratio. Saline only (SO, n=11), MPTP only (MO, n=7), and MPTP + exercise (ME, n=10). Panel B: Corticostriatal afferents were stimulated in vitro at the same stimulus intensity in all brain slices. The examples to the left illustrated the reduction in the size of AMPA receptor-mediated synaptic responses with exercise in the MPTP lesioned mouse. The graph to the right illustrates the input (stimulus intensity) – output (excitatory postsynaptic current or EPSC) relationship for MPTP alone (n=7) and MPTP exercise (n=6) groups.



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Neurobiology of Disease 32 (2008) 319–327

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Memory, mood, dopamine, and serotonin in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse model of basal ganglia injury

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ARTICLE INFO

Article history:
Received 19 April 2008
Revised 7 July 2008
Accepted 25 July 2008
Available online 5 August 2008

Keywords:
Affective behavior
MPTP
Parkinson's disease
Anxiety
Depression
Conditioned fear

ABSTRACT

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mouse serves as a model of basal ganglia injury and Parkinson's disease. The present study investigated the effects of MPTP-induced lesioning on associative memory, conditioned fear, and affective behavior. Male C57BL/6 mice were administered saline or MPTP and separate groups were evaluated at either 7 or 30 days post-lesioning. In the social transmission of food preference test, mice showed a significant decrease in preference for familiar food 30 days post-MPTP compared to controls. Mice at both 7 and 30 days post-MPTP lesioning had increased fear extinction compared to controls. High Performance Liquid Chromatography analysis of tissues homogenates showed dopamine and serotonin were depleted in the striatum, frontal cortex, and amygdala. No changes in anxiety or depression were detected by the tail suspension, sucrose preference, light–dark preference, or hole-board tests. In conclusion, acute MPTP lesioning regimen in mice causes impairments in associative memory and conditioned fear, no mood changes, and depletion of dopamine and serotonin throughout the brain.

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by motor impairment including slowness of movement, rigidity, balance dysfunction, and resting tremor. However, disabling non-motor symptoms are seen in 30% to 60% of patients and include semantic and episodic memory loss, impairment of executive function, depression, and anxiety (Cummings, 1992; Hornykiewicz, 1963; Pillon et al., 1989a; Walsh and Bennett, 2001). A number of brain regions have been implicated in influencing non-motor behavioral symptoms including the basolateral amygdala, nucleus accumbens, frontal cortex, and the raphe nucleus (Ressler and Nemeroff, 2000; Walsh and Bennett, 2001). Together with dopamine, serotonin from the dorsal and medial raphe nuclei is thought to play a central role in regulating affective behavior. Perturbation of serotonin neurotransmission in normal individuals can lead to depression, anxiety, and memory impairment (Mann and Yates, 1986; Mann, 1999; Pillon et al., 1989b). Patients with PD develop central serotonergic dysfunction, such as low cortical serotonin levels and degeneration of the dorsal raphe nucleus (Agid et al., 1989; Cummings, 1992; Gotham et al., 1986; McCance-Katz et al., 1992; Scatton et al., 1983). 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) neurotoxicity in the substantia nigra pars compacta (SNpc) produces severe dopamine depletion in mice and nonhuman primates, and causes significant decrease of serotonin across multiple brain regions. For example, chronic MPTP treatment in monkeys decreases levels of serotonin in the caudate nucleus, putamen, nucleus accumbens, hypothalamus, and cortical areas (Frechilla et al., 2001; Perez-Otano et al., 1991; Pifl et al., 1991; Russ et al., 1991). In mice, acute administration of MPTP leads to serotonin loss in the striatum and frontal cortex one week after lesioning (Rousselet et al., 2003).

The purpose of the current study was to evaluate the effects of MPTP lesioning on associative memory, conditioned fear, depression, and anxiety, since this neurotoxic injury leads to depletion of dopamine and serotonin in brain regions important for these behaviors. After acute MPTP lesioning, mice were tested at 7 days (greatest dopamine depletion) and 30 days (partial recovery of striatal dopamine). We used established mouse tests for associative memory (social transmission of food preference), fear conditioning, anxiety (light–dark preference, hole board), and depression (tail suspension, sucrose preference). Brain regions involved in control of affective behavior (frontal cortex, amygdala, and the raphe nucleus), as well as the basal ganglia (ventral mesencephalon, striatum), were examined

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Available Online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2008.07.015

for levels of dopamine, serotonin and their metabolites. We observed impairment in associative memory in mice at 30 days post-MPTP lesioning and increased fear extinction at both 7 and 30 days post-MPTP. Despite significant depletion of both dopamine and serotonin at these time points, there was no significant increase in depression and anxiety compared to control mice. Overall, these results indicate that the acute MPTP-lesioned mouse model manifests some but not all non-motor behaviors seen in patients with PD.

Materials and methods

Animals, treatment groups and MPTP administration

Male C57BL/6 mice, 8 to 10 weeks of age (Charles River Laboratories, Wilmington, MA) and weighing between 25 and 30 g were group-housed in a temperature-controlled room under a 12-h light/12-h dark cycle with free access to water and standard rodent food. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at the University of Southern California. A total of 42 mice were used in this study. For lesioning, mice received 4 i.p. injections of 20 mg/kg MPTP (free-base; Sigma-Aldrich, St. Louis, MO) in saline at 2-h intervals or 4 injections of 0.1 ml 0.9 % NaCl as control. Mice were tested either at (i) 7 days post-saline (n=20), (ii) 7 days post-MPTP (n=10) or (iii) 30 days post-MPTP (n=12). The degree of lesioning was determined at both 7 and 30 days post-lesioning using unbiased stereological counting of the remaining dopaminergic neurons in the SNpc as well as analysis of the immunoreactivity for tyrosine hydroxylase protein within the striatum. These methods are outlined below and the degree of lesioning was in agreement with previous reports using the same MPTP lesioning regimen (Jackson-Lewis et al., 1995; Jakowec et al., 2001; Petzinger et al., 2001; Petzinger et al., 2007; Przedborski et al., 2001).

Behavioral testing

After MPTP lesioning, mice were subjected to a series of behavioral tests for anxiety (light–dark exploration, hole-board), depression (sucrose preference, tail suspension), associative memory (social transmission of food preference [STFP]), and conditioned fear. Tests were conducted over 6 days with the following order: (1) STFP, (2) light–dark exploration, (3) sucrose preference, (4) hole-board, (5) conditioned odor aversion, (6) tail suspension, and (7) conditioned fear (Fig. 1). The design of the behavior testing battery took into account starting with the least stressful test and progressing to the most stressful (Crawley, 2008). The tests which are known to induce the most acute stress response (tail suspension and fear conditioning) (Brown et al., 1984; Liu et al., 2003; Pugh et al., 1997) were conducted at the end of the battery. The order of tests was not randomized. Any potential carry-over effects were equal between the groups as all mice were tested in the same order. Tests occurring on the same day were conducted at least 3 h apart. Each behavioral test was administered once to each mouse. All tests were performed in a darkened room with dim red lights and animals were allowed to habituate to the testing room for 1 h prior to each test. Details for each test are presented in the following sections.

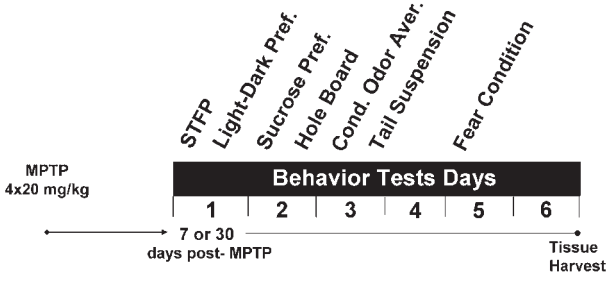

Fig. 1. Diagram representing the timeline and order of behavioral tests following acute MPTP lesioning (4×20 mg/kg, 2 h apart) or saline injections (4×0.1 ml, 2 h apart). The order of behavioral tests was designed according to increased stress. Separate groups of mice were tested at 7 or 30 days post-MPTP lesioning.

Social transmission of food preferences (STFP) for olfactory memory was conducted as described previously (Holmes et al., 2002; Kogan et al., 1997; Wrenn et al., 2003). Briefly, a demonstrator mouse was randomly chosen from each home cage prior to MPTP administration. Initially, all mice were habituated for 18 h to powdered chow presented in two 125-g food jar assemblies (Dyets, Inc., Bethlehem, PA) in the opposite corners of the home cage. During this time, standard food pellets were unavailable. One day later, demonstrator mice were removed from their home cages, individually housed, and food-deprived overnight with free access to water. The next day, each demonstrator mouse received powdered chow mixed with either 1% cinnamon or 2% cocoa (w/w) for 1 h, or until at least 0.2 g of powdered food was consumed. To avoid a bias in the cued flavor, half of the demonstrators randomly received cinnamon- and the other half cocoa-flavored food. Immediately afterwards, demonstrator mice were returned to their home cages to interact with observer mice for 30 min. At the end of the interaction period, demonstrator mice were removed. Testing of food preference in observer mice took place 24 h later, following overnight food deprivation with free access to water. During the test, observer mice were caged individually and were given a free choice of food flavored with 1% cinnamon or 2% cocoa. To control for possible place preference, the position of the food jar assemblies with the cued flavor was balanced between cages. Observer mice were allowed to eat for 1 h and food consumption from each jar was determined by weight. The percent of total food intake consumed as the cued flavor was determined.

The light–dark exploration test for anxiety was conducted as previously described (Holmes et al., 2002). The test uses the ethological conflict between the tendencies of mice to explore a novel environment and to avoid a brightly lit open area. This test has been shown to be sensitive to changes in serotonergic tone (Holmes et al., 2002). A standard polypropylene mouse cage (30×19×13 cm) was divided with an opaque partition containing a small opening at the bottom (8×5 cm) into a larger light chamber and a smaller dark chamber. The light chamber (20×19×13 cm) was transparent and brightly illuminated by a 60-W bulb placed 40 cm above the cage top. The dark chamber (10×19×13 cm) was black and closed at the top with a black Plexiglas lid. The test was conducted in a soundproof room and the apparatus was cleaned with warm water and 70% ethanol between each mouse. Each mouse was placed in the lighted chamber facing away from the entrance to the dark chamber, and its behavior was recorded on video for 5 min. Measurements were obtained for: (i) latency to first enter the dark chamber, (ii) time spent in the dark, and (iii) number of transitions between the two compartments. A transition was considered only when a mouse entered into a compartment with 3 or more paws.

The sucrose preference test for depression was performed as a modification of the 2-bottle preference test for mice (Strekalova et al., 2004). Mice were deprived of food and water overnight and placed in separate cages 1 h before the start of testing. Mice were offered solutions of 1% sucrose (Sigma-Aldrich, MO) or tap water for 1 h. Fluid consumption was determined by weight and expressed as percent of total fluid intake consumed from the sucrose solution. The positions of the water and sucrose bottles were alternated to control for side preferences.

The hole-board test for anxiety based on exploratory behavior was performed as previously described (Boissier and Simon, 1962; do-Rego et al., 2006). This test is frequently used as an indicator of directed

exploratory behavior in rodents (Crawley, 1985). The testing apparatus consisted of a 2-cm-thick square plastic board, 40×40 cm, with 16 holes (2 cm diameter) regularly spaced 7 cm apart over the surface and 3.5 cm from the edges (Ugo Basile, Italy). The board was positioned 50 cm above floor level. Each animal was placed at the corner of the board and allowed to freely explore for 5 min. The number and location of head dips was recorded using a video camera and videotapes were scored by a trained observer blinded to the treatment group. A head dip was considered when a mouse placed its head into a hole up to the neck. Between testing of each mouse, the board surface was cleaned with water and 70% ethanol.

The conditioned odor aversion with isoamyl-acetate was used as a rapid assessment of odor detection in mice. The one-bottle test was performed as previously described (Passe and Walker, 1985; Wright and Harding, 1982) with minor modifications. Mice were deprived of food and water overnight and housed separately 1 h before the start of testing. Testing consisted of four 10-min trials. Breaks between the trials lasted 30 min. During the first two trials, mice were allowed to drink water containing both 0.1% (v/v) isoamyl acetate (artificial banana aroma) and 0.5% (w/v) quinine hydrochloride (bitter taste) (Sigma-Aldrich, St. Louis, MO). During the third trial, mice were tested for avoidance of isoamyl acetate odorized water without quinine hydrochloride. The last trial consisted of tap water. Fluid consumption was determined by weight. Preference ratio for isoamyl acetate was determined from the last two trials as follows: odorized water (g)/odorized water+tap water (g). Preference ratio of below 0.5 indicates aversion, and therefore detection of the odorant.

The tail suspension test for depression was preformed as previously described (Steru et al., 1985). This test relies on immobility as a measure of "behavioral despair" once the mouse perceives that the escape from the apparatus is impossible. Mice were individually suspended by their tails at a height of 20 cm using a piece of adhesive tape wrapped around the tail, 2 cm from the tip. Behavior was videotaped for 6 min. The duration of immobility was measured using a stopwatch. Mice were considered immobile only when hanging completely motionless. Mice that climbed up their tails were excluded from analysis. Results were expressed as percent of time spent immobile.

Auditory conditioned fear response was assessed as previously described (LeDoux, 2000). The test consisted of an 8-min acquisition phase on the first day and an 8-min extinction phase on the next day. Training was conducted in a soundproof room with dim red light and background noise level of 50 dB. Each mouse was placed in the middle of a testing chamber (23×20×20 cm) with an electrified metal rod floor (2 mm diameter, 6 mm separation). The chamber was cleaned with water and 70% ethanol before testing each mouse. The fear acquisition consisted of a 3-min acclimation period, 3 pairings of tone/foot shock separated with 1-min quiet intervals, and 1-min quiet consolidation period at the end of testing. For each pairing of tone/foot shock, mice were presented tone (30 s of 80dB, 1000 Hz/8000 Hz continuous alternating sequence of 250 ms pulses) generated using LabView 7.1 software (National Instruments Corporation, Austin, TX) and delivered through speakers on the top of the testing chamber. Each tone was immediately followed by a mild foot shock (2 s, 0.6 mA). Freezing behavior (no visible movement except for respiration) was recorded on videotape during the test. The extinction phase was conducted the next day in a different room illuminated with blue indirect light. Each mouse was placed in a cylindrical Plexiglas observation chamber (diameter 28 cm) with a smooth Plexiglas floor. Following an initial 2 min acclimation period, recall and extinction of freezing in response to the tone (presented continuously for 6 min) was monitored in the absence of foot shock. The duration of the freezing response in seconds was measured within each 1-min interval of both acquisition and extinction phases. Freezing behavior was manually scored using Observer XT version 6.1.35 software (Noldus Information Technology, San Diego, CA).

Tissue preparation

Brains were collected 24 h after the last behavior test. For immunohistochemistry, a subset of mice (n=4 per group), were sacrificed by pentobarbital overdose and transcardially perfused with 4% paraformaldehyde, post-fixed in the perfusion fixative for 24 h at 4 °C, cryoprotected in 20% sucrose for 48 h, frozen in isopentane on dry ice, and stored at −80 °C. For HPLC analysis, another subset of mice (n=5 per group) were killed by cervical dislocation. Brains were quickly removed and regions of interest identified using a standard mouse brain atlas (Paxinos and Franklin, 2001). Frontal cortex (rostral to Bregma +2.50), dorsal and ventral regions of mid-striatum (including the nucleus accumbens), amygdala, ventral mesencephalon (containing substantia nigra and VTA) and the raphe nucleus (dorsal and medial part) were rapidly dissected, immediately frozen in isopentane on dry ice and stored at −80 °C.

Neurochemical analysis

Neurotransmitter concentrations of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin, and 5-hydroxyindoleacetic acid (5-HIAA) were determined by HPLC with electrochemical detection as previously described (Irwin et al., 1992; Kilpatrick et al., 1986; Petzinger et al., 2007). The system consisted of an ESA auto-sampler (ESA Inc., Chelmsford, MA) equipped with a 150×3.2 mm reverse phase C-18 column (3μm diameter) regulated at 28 °C and a CoulArray 5600A (ESA Inc, Chelmsford, MA), equipped with a 4-channel analytical cell with potentials set at −100 mV, −30 mV, 220 mV and 350 mV. The HPLC was integrated with a DellGX-280 computer with CoulArray analytical program for Windows (ESA Inc, Chelmsford, MA). Mobile phase consisted of acetonitrile in phosphate buffer and an ion-pairing agent and was delivered at a rate of 0.6 ml/min. Fresh frozen tissue was homogenized in 0.4 M $HClO_4$, and centrifuged to separate precipitated protein. The pellet was resuspended in 0.5 M NaOH and used to determine total protein concentration with the CoomassiePlus protein assay (Pierce, Rockford, IL) and microplate reader ELx800 (BioTek Instruments Inc., Winooski, VT) equipped with KCjunior software.

Immunohistochemical staining

Analysis of relative expression of striatal tyrosine hydroxylase (TH) immunoreactivity was carried out as previously described (Jakowec et al., 2004; Petzinger et al., 2006; Petzinger et al., 2007). Briefly, coronal brain sections were cut at 25-μm thickness through the mid-striatum and collected in phosphate-buffered saline (PBS, pH 7.2). Sections were exposed to rabbit polyclonal anti-tyrosine hydroxylase antibody (1:5000, Chemicon, Temecula, CA) for 24 h at 4 °C followed by 2 h incubation in IRDye700 conjugated goat anti-rabbit IgG (1:2500, Molecular Probes, Eugene, OR). Following extensive washing, sections were mounted on gelatin-coated slides and scanned using LI-COR Odyssey near infrared imaging platform (LI-COR Biotechnology, Lincoln, NE). Multiple brain sections at the mid-striatum (4 to 5 sections per mouse) from 4 mice per group were prepared and analyzed in parallel. Fluorescence intensity within an oval shaped region of interest (0.5 mm^2) in dorsal striatum was measured and corrected for background by subtracting the adjacent corpus callosum. Values for treatment groups were normalized to saline animals prior to statistical analysis.

Unbiased stereological counting of SNpc dopaminergic neurons

The degree of MPTP lesioning at 7 and 30 days post-MPTP treatment was determined by unbiased stereological counting of dopaminergic neurons in the SNpc. For this purpose, coronal sections were collected starting rostral to the substantia nigra at Bregma

−2.50 mm before the closure of the third ventricle through to the prominence of the pontine nuclei at Bregma −4.24 mm according to the stereotaxic atlas of the mouse brain (Paxinos and Franklin, 2001). Every sixth section from 4 mice per group was included in the analysis. Sections were exposed to rabbit polyclonal anti-tyrosine hydroxylase antibody (1:5000, Chemicon, Temecula, CA) for 24 h at 4 °C followed by and avidin–biotin complex (ABC elite Kit, Vector Labs, Burlingame, CA). Staining was visualized by exposure to 3,3′ diaminobenzidine tetrahydrochloride (Pierce, Rockford, IL), after which sections were mounted on gelatin-coated slides, and cover-slipped. Cell nuclei were visualized by cresyl-violet staining. The SNpc was delineated from the rest of the brain based on TH-ir. Sections were examined using an Olympus BX-50 microscope (Olympus Optical, Tokyo, Japan) equipped with a motorized stage and digital Retiga cooled CCD camera (Q-Imaging, Burnaby, British Columbia, Canada). Each stained ventral mesencephalon section was viewed at low magnification (4×) and the SNpc outlined and delineated from the ventral tegmental-immunoreactive neurons using the third nerve and cerebral peduncle as landmarks. Neurons were viewed at higher magnification (40×) and counted if they displayed TH-ir and had a clearly defined nucleus, cytoplasm, and nucleolus. Analysis was performed with the computer-imaging program BioQuant Nova Prime (BioQuant Imaging, Nashville, TN). The total number of SNpc dopaminergic neurons was determined based on the method of Gundersen and Jensen (1987).

Statistical analysis

With the exception of fear-conditioning, all results were evaluated by a one-way analysis of variance (ANOVA) and Bonferroni multiple comparison test when appropriate, or by one-way ANOVA on ranks followed by Dunn's post-hoc test when the normality test or equal variances test failed. Software used for statistical analysis was Prism5 for Windows (Graph Pad Software Inc., San Diego, CA). Data from all experiments are presented as mean ± SEM and $p<0.05$ was considered significant.

Results

Social transmission of food preference

Associative olfactory memory was assessed using the STFP test (Fig. 2). When presented with 2 unfamiliar flavors of powdered food, control mice strongly preferred the flavor previously consumed by the demonstrator mouse (79.0 ± 3.7% of total food intake). Lesioned mice tested 7 days after MPTP showed a similar preference (79.0 ± 3.3%). However, preference for the demonstrated flavor declined significantly in mice tested 30 days post-MPTP (58.7 ± 6.3%) ($F_{(2,41)}=5.614$; $p<0.05$). As the STFP test relies on the ability of mice to discriminate odors, it was important to test if all mice had similar olfactory function. For this purpose, the conditioned odor aversion test with isoamyl acetate was used. Mice from all three groups avoided water odorized with isoamyl acetate (preference ratio for saline: 0.4 ± 0.1; 7 days post-MPTP: 0.2 ± 0.1; 30 days post-MPTP: 0.3 ± 0.1). Preference ratio below 0.5 indicates the ability to discriminate odors. Based on these results, mice in this study did not display detectable impairment in olfactory function.

Light–dark preference and hole-board

Light–dark preference and the hole-board tests were used to determine levels of anxiety in mice 7 and 30 days post-MPTP lesioning (Fig. 3). Mice from all three groups showed a similar preference for the dark compartment ($F_{(2,39)}=1.428$; $p>0.05$) during the light–dark exploration test (Fig. 3A). Saline-treated mice spent 66.1 ± 3.7% of the time in the dark. Likewise, mice at 7 days post-MPTP lesioning spent 74.5 ± 3.6% of time in the dark and those tested at 30 days post-

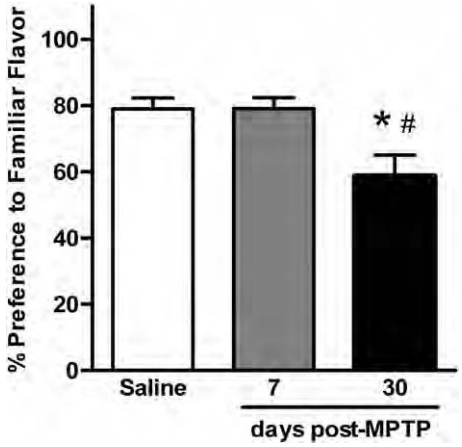

Fig. 2. Associative memory impairment in MPTP-lesioned mice measured by the social transmission of food preference test. The preference for familiar food was tested in control ($n=20$) and lesioned mice at 7 days ($n=10$) and 30 days post-MPTP ($n=12$). Data are presented as mean ± SEM of percent preference for familiar food. The symbols "*" and "#" represent statistically significant differences compared to the saline control and the 7 days post-MPTP groups, respectively ($p<0.05$).

MPTP lesioning spent 65.4 ± 3.3% of the time in the dark. The average number of transitions between the light and dark compartments was similar in all three groups (13.1 ± 1.6 for saline mice; 12.6 ± 1.3, and 16.0 ± 1.9 for mice 7 days and 30 days post-MPTP lesioning, respectively; $F_{(2,39)}=1.246$; $p>0.05$). There was no significant difference between the latency to first enter the dark compartment between groups (26.7 ± 5.3 s for saline mice; 15.9 ± 4.2 s and 25.6 ± 5.6 s for mice 7 days and 30 days post-MPTP lesioning, respectively; $F_{(2,39)}=0.9846$; $p>0.05$). Exploratory behavior was measured in the hole-board test as a second test of anxiety (Fig. 3B). As measured by head dipping, saline-treated mice visited 24.8 ± 2.2 holes during the 5-min test, similar to mice at 7 days post-MPTP lesioning (24.5 ± 3.0 holes). Mice tested at 30 days post-MPTP lesioning had 33.0 ± 3.1 head dips. The difference in the total number of head dips between groups was not statistically significant ($F_{(2,33)}=3.053$; $p>0.05$).

Sucrose preference and tail suspension

Sucrose preference and the tail suspension tests were used to determine levels of depression in mice after MPTP lesioning (Fig. 4). In particular, the sucrose preference test measures anhedonia following overnight water deprivation (Strekalova et al., 2004). Mice from all three groups had high preference for a 1% sucrose solution (80–85%) compared to tap water (15–20%) (Fig. 4A). There was no significant difference in the amount of sucrose consumed ($F_{(2,38)}=0.968$; $p>0.05$). Furthermore, all mice had similar fluid intake during the 1-h testing period (saline mice: 0.9 ± 0.1 g; 7 days post-MPTP lesioning: 0.8 ± 0.1 g; and 30 days post-MPTP lesioning: 1.1 ± 0.1 g; $F_{(2,37)}=1.268$; $p>0.05$). The tail suspension test measures behavioral despair (Steru et al., 1985). Saline-treated mice spent 36.6 ± 3.1% of total time in passive immobility (Fig. 4B). Similarly, mice at 7 days post-MPTP lesioning spent 29.3 ± 3.3% of total time in immobility. Interestingly, mice tested at 30 days post-MPTP lesioning spent significantly more time immobile (44.3 ± 3.2%) compared to the 7 days post-MPTP group ($F_{(2,39)}=4.372$; $p<0.05$); however, this was not statistically different compared to saline-treated mice. Only one mouse in the control group was excluded from the test because it climbed up its tail. None of the MPTP-lesioned mice were excluded from the test.

Conditioned fear

Acquisition and extinction of conditioned fear response was measured using the auditory fear conditioning test. All mice showed

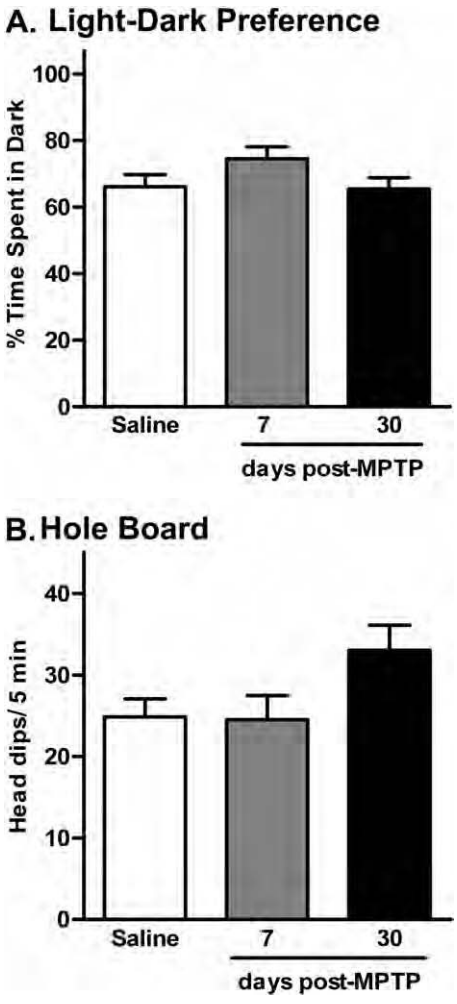

Fig. 3. Anxiety in mice 7 and 30 days post-MPTP lesioning measured in light–dark preference and the hole-board tests. Data are presented as mean±SEM from control (n=20), 7 days (n=10) and 30 days post-MPTP (n=12). (A) Time spent in the dark compartment during the 5-min light–dark preference test (in percent of total time). (B) The total number of head dips during the 5-min hole-board test.

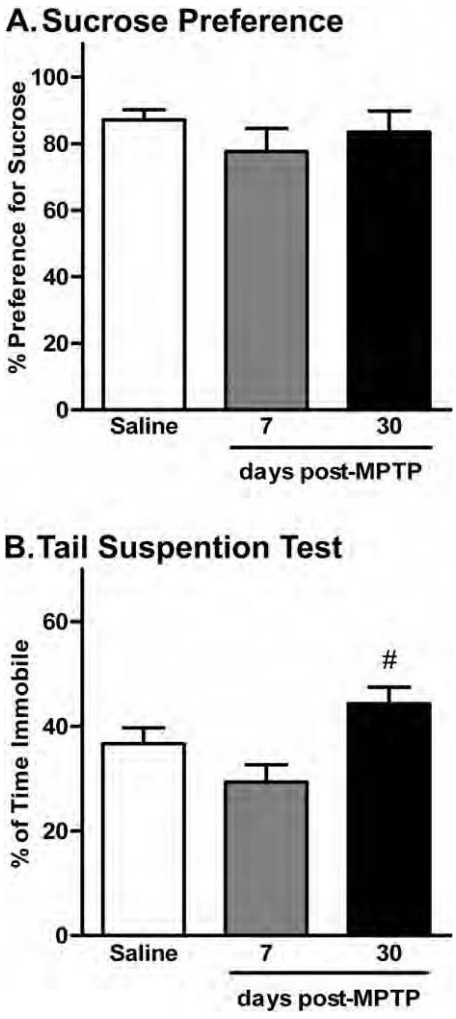

Fig. 4. The effect of acute MPTP lesioning on depression in mice 7 and 30 days post-treatment measured using sucrose preference and tail suspension tests. Data are presented as mean±SEM of (A) preference for 1% sucrose and (B) time spent in immobility (in percent of total time) for control (n=18), 7 days (n=10) and 30 days post-MPTP (n=12). The symbol "#" represents statistically significant difference compared to the 7 days post-MPTP group (p<0.05).

little or no freezing during the baseline period of acquisition session (percent time freezing for saline: 1.4±0.4%; for 7 days post-MPTP: 0.2±0.1%; and for 30 days post-MPTP: 0.7±0.4%). During subsequent pairings of tone and foot shock, all mice showed increased freezing behavior. Mice in all three groups had similar levels of freezing after the third foot shock (saline: 54.9±10.7%; 7 days post-MPTP: 46.2±10.5%; and 30 days post-MPTP: 49.4±4.1%; $F_{(2,16)}$=0.249; p>0.05). The next day (Fig. 5), the baseline freezing response of all mice was similar (percent freezing for saline: 23.7±6.2%; 7 days post-MPTP: 9.6±2.4%; and 30 days post-MPTP: 18.5±8.3%). At the onset of auditory stimulus (without foot shock), all mice showed a robust increase in the freezing response (saline: 68.8±3.1%; 7 days post-MPTP: 56.8±6.7%; and 30 days post-MPTP: 51.0±6.2%). However, after 6 min of continuous tone exposure, both groups of MPTP-lesioned mice spent significantly less time freezing (7 days post-MPTP: 9.5±3.2%; and 30 days post-MPTP: 17.5±8.2%) compared to saline controls (43.7±6.0%) ($F_{(2,17)}$=23.08; p<0.05).

HPLC analysis of dopamine, serotonin, and their metabolites

HPLC analysis was used in tissue homogenates to determine the levels of dopamine and its metabolites (DOPAC and HVA), as well as serotonin and its metabolite 5-HIAA. The turnover ratio for dopamine was determined as ([DOPAC]+[HVA]) / [dopamine] and for serotonin as [5-HIAA]/[serotonin]. Six brain regions were analyzed including the frontal cortex (rostral to the motor cortex), dorsal striatum, ventral striatum (including the nucleus accumbens), ventral mesencephalon (VME) (including the substantia nigra and VTA), amygdala, and the

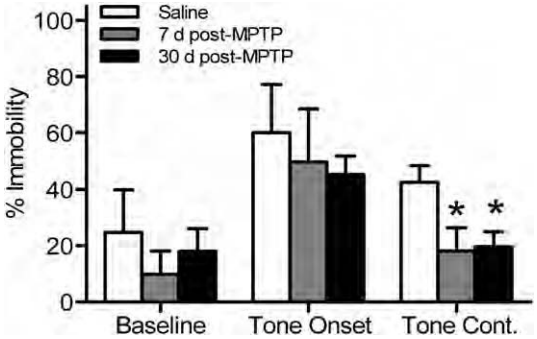

Fig. 5. Extinction of fear response in MPTP-lesioned mice measured in the fear conditioning test. Fear-induced immobility was measured in control (n=5), 7 days (n=6), and 30 days post-MPTP mice (n=8). Data are presented as mean±SEM of the freezing response (percent of 2- or 4-min periods). Following 2 min baseline, continuous tone was played for 6 min without foot shock (tone onset: min 3–4; tone continuation: min: 5–8). The symbol "*" indicates statistically significant difference compared to the saline control group (p<0.05).

raphe nucleus (including the dorsal and medial raphe) (Table 1 and Fig. 6). Taken together, MPTP-lesioned mice had severe dopamine depletion in the dorsal and ventral striatum and frontal cortex, and no significant loss in the amygdala, VME, or the raphe nucleus. The greatest depletion of dopamine was measured at 7 days post-MPTP. Serotonin was significantly depleted in the dorsal and ventral striatum, frontal cortex, and amygdala. The greatest loss of serotonin was measured at 30 days post-MPTP lesioning. There was no significant change in the level of the serotonin metabolite 5-HIAA in any of the examined brain regions.

Among all six regions investigated, the dorsal striatum of saline-treated mice contained the highest concentration of dopamine (141.3±12.4 ng dopamine/mg protein). Here, acute MPTP lesioning caused a significant loss of dopamine that persisted for at least 30 days ($F_{(2,14)}$=89.00; p<0.05). There was 95% depletion in mice at 7 days post-MPTP (7.5±1.6 ng dopamine/mg protein) and 86% depletion at 30 days post-MPTP lesioning (19.9±5.2 ng dopamine/mg protein). The change in dopamine turnover ratio did not reach significance at 7 days (1.1±0.4) nor at 30 days post-MPTP lesioning (1.4±0.3) compared to controls (0.2±0.0) ($F_{(2,14)}$=3.654; p>0.05). The ventral striatum of saline-treated mice contained the second highest concentration of dopamine (91.0±7.7 ng dopamine/mg protein). In this region MPTP caused an 86% depletion at 7 days post-MPTP lesioning (13.0±1.3 ng dopamine/mg protein) and 77% depletion at 30 days post-MPTP (21.6±6.1 ng dopamine/mg of protein) ($F_{(2,14)}$=56.63; p<0.05). However, differences in dopamine turnover ratio were not statistically significant ($F_{(2,14)}$=3.654; p>0.05), similar to dopamine turnover in the dorsal striatum.

In the frontal cortex of control mice the dopamine concentration (4.0±1.5 ng dopamine/mg protein) was low compared to the striatum. Nonetheless, MPTP lesioning caused significant dopamine loss (88% depletion) at 7 days after MPTP (0.5±0.1 ng dopamine/mg protein) and at 30 days post-MPTP (0.6±0.2 ng dopamine/mg protein; 86% depletion) ($F_{(2,14)}$=5.43; p<0.05). Furthermore, dopamine turnover ratio was significantly increased at 7 days (1.4±0.2) and 30 days (4.2±1.3) post-MPTP lesioning, compared to controls (0.8±0.2) ($F_{(2,14)}$=5.863; p<0.05).

Table 1
Dopamine and serotonin levels measured by HPLC and their calculated turnover ratios in six brain regions of the MPTP-lesioned and control mice

	Treatment	DA	DA Turnover	5-HT	5-HT Turnover
Dorsal Striatum	Control	141.3±12.4	0.2±0.0	7.1±0.8	1.0±0.1
	7 days post-MPTP	7.5±1.6*	1.1±0.5	3.7±0.5*	2.4±1.0
	30 days post-MPTP	19.9±5.2*	1.4±0.3	3.3±0.4*	2.9±0.5
Ventral Striatum	Control	91.1±7.7	0.2±0.0	16.4±1.8	0.7±0.1
	7 days post-MPTP	13.0±1.3*	0.6±0.2	10.0±0.9*	1.1±0.4
	30 days post-MPTP	21.6±6.1*	1.2±0.4	9.1±1.6*	1.9±0.6
Frontal Cortex	Control	4.0±1.5	0.8±0.2	12.8±1.1	0.5±0.0
	7 days post-MPTP	0.5±0.1*	1.4±0.2	6.7±1.5*	0.8±0.3
	30 days post-MPTP	0.6±0.2*	4.2±1.3*	5.1±1.0*	2.5±1.0
Amygdala	Control	9.2±2.5	0.7±0.1	11.9±0.8	1.2±0.2
	7 days post-MPTP	4.8±1.0	0.8±0.3	8.6±1.6	1.9±0.8
	30 days post-MPTP	3.5±1.0	2.1±1.0	6.8±1.3*	3.2±1.2
VME	Control	5.2±0.8	1.1±0.0	23.7±0.7	1.1±0.0
	7 days post-MPTP	5.1±1.2	1.8±0.6	20.7±4.9	2.8±1.2
	30 days post-MPTP	3.8±2.0	4.6±1.4*	12.9±5.2	6.6±2.4
Raphe Nucleus	Control	1.4±0.2	1.0±0.0	15.8±2.0	1.6±0.4
	7 days post-MPTP	1.9±0.2	0.8±0.1	12.5±3.5	3.2±1.5
	30 days post-MPTP	1.5±0.2	1.2±0.3	11.5±2.9	3.3±1.1

HPLC analysis of dopamine, serotonin, and their calculated turnover ratios in the dorsal striatum, ventral striatum, frontal cortex, amygdala, ventral mesencephalon (VME), and raphe nucleus from control, 7 days, and 30 days post-MPTP-lesioned mice (n=5/group). Data are presented as mean±SEM of monoamine concentrations (in ng/mg of protein). Abbreviations: DA—dopamine; 5-HT—serotonin. Dopamine turnover ratio was calculated as: ([DOPAC]+[HVA])/[DA] and serotonin turnover ratio as: [5-HIAA]/[5-HT]. The symbol "*" indicates statistically significant differences compared to the saline control group (p<0.05).

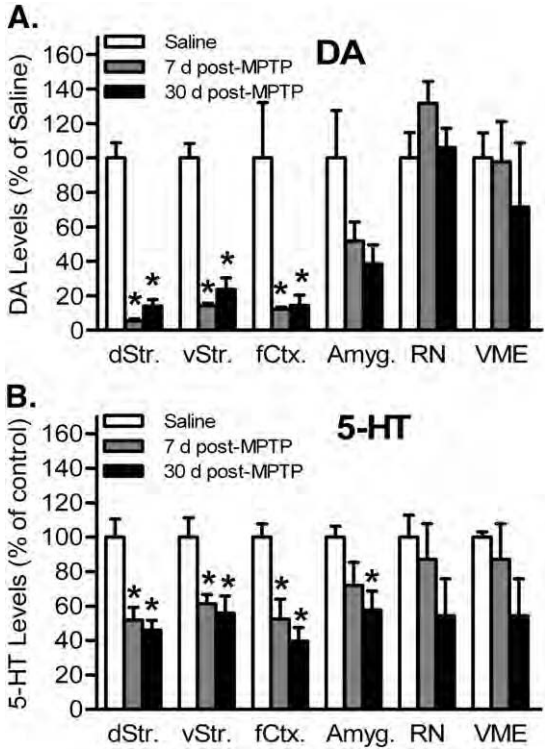

Fig. 6. Dopamine and serotonin levels in MPTP-lesioned mice. Twenty-four hours following behavioral testing, the dorsal striatum (dStr), ventral striatum (vStr), frontal cortex (fCtx), amygdala, ventral mesencephalon (VME), and the raphe nucleus (RN) tissue homogenates were analyzed for monoamine concentrations (n=5/group) using HPLC. Data are presented as mean±SEM. (A) Dopamine and (B) serotonin loss relative to control mice in brain regions regulating associative memory and affective behavior at 7 and 30 days post-MPTP lesioning. The symbol "*" indicates statistically significant differences compared to the saline control group (p<0.05).

In the amygdala of saline-treated mice dopamine levels (9.2±2.5 ng dopamine/mg protein) were about one tenth of those in the striatum. MPTP-induced dopamine loss in amygdala did not reach statistical significance ($F_{(2,14)}$=3.132; p>0.05), and dopamine turnover ratio remained similar between the groups (Table 1). Cell bodies of dopamine and serotonin-producing neurons are located in VME and the raphe nucleus, respectively. These two regions had low detectable levels of dopamine in control mice (Table 1) but MPTP lesioning did not cause statistically significant dopamine loss in the VME nor the raphe nucleus (Fig. 6A). The fact that the VME also contained the VTA, which is less affected by MPTP lesioning, accounts for the fact that changes in dopamine within this region were not statistically different from saline control at 7 and 30 days post-lesioning.

HPLC analysis of serotonin in tissue homogenates showed that loss of this neurotransmitter was modest compared to dopamine loss following MPTP lesioning. In the dorsal striatum of saline control mice, serotonin was low (7.1±0.8 ng serotonin/mg protein) but nonetheless, was significantly depleted at 7 days (3.7±0.5 ng serotonin/mg protein, 48% loss) and at 30 days post-MPTP lesioning (3.3±0.4 ng serotonin/mg protein, 64% loss) ($F_{(2,14)}$=13.10; p<0.05). The ventral striatum of control mice contained twice as much serotonin compared to the dorsal striatum (16.4±1.8 ng serotonin/mg protein). Here, the serotonin loss was moderate but still significant: 10.0±0.9 ng/mg protein or 39% depletion at 7 days and 9.1±1.6 ng serotonin/mg protein or 44% depletion at 30 days post-MPTP lesioning respectively ($F_{(2,14)}$=6.80; p<0.05). The serotonin turnover ratio in both the dorsal and ventral striatum did not change significantly following MPTP lesioning (Table 1).

Serotonin concentrations in the frontal cortex (12.8±1.1 ng serotonin/mg protein) and amygdala (11.9±0.7 ng serotonin/mg

protein) were similar in control mice. MPTP lesioning caused a 48% depletion in the frontal cortex at 7 days (to 6.7±1.5 ng serotonin/mg protein) and 60% depletion at 30 days post MPTP lesioning (to 5.1±1.0 ng serotonin/mg protein). This decrease was statistically significant compared to controls ($F_{(2,14)}$=11.28; p<0.05). There was no change in serotonin turnover ratio in the frontal cortex (Table 1). In the amygdala, serotonin remained unchanged at 7 days post-MPTP (8.5±1.6 ng serotonin/mg protein); however, it was significantly depleted at 30 days post-MPTP lesioning (6.9±1.3 ng serotonin/mg protein) compared to control ($F_{(2,14)}$=4.03; p<0.05).

The raphe nucleus (containing both the dorsal and medial aspects) and VME (containing the substantia nigra and VTA) had high average serotonin concentrations in all mice (Table 1) and MPTP lesioning did not cause a significant depletion of serotonin in these regions.

TH immunoreactivity in dorsal striatum and dopaminergic cell loss in SNpc

TH immunoreactivity is often used as a marker of the integrity of dopaminergic axons in the striatum (Jakowec et al., 2004; Petzinger et al., 2007). There was significant reduction of TH-immunoreactivity (TH-ir) in dorsal striatum of mice 7 and 30 days post-MPTP lesioning ($F_{(2,9)}$=45.41; p<0.05). MPTP caused a 58% reduction of TH-ir in the dorsal striatum examined at 7 days post-lesioning and 45% loss at 30 days post-MPTP lesioning.

The number of surviving TH-positive neurons in the SNpc was used as an additional measure of the integrity of midbrain dopaminergic system 7 and 30 days post-MPTP lesioning. MPTP lesioning caused 68% loss of TH-ir SNpc neurons in 7 days post-MPTP and 66% loss at 30 days post-MPTP.

Discussion

The MPTP-lesioned mouse serves as a model of basal ganglia injury and Parkinson's disease. While the majority of studies focus specifically on motor deficits, few studies have addressed the non-motor features including affective behavior. The purpose of this study was to examine non-motor behaviors (associative memory, conditioned fear, anxiety, and depression) in the C57BL/6 mouse following a standardized acute lesioning regimen with MPTP (Jackson-Lewis et al., 1995). We report associative memory impairment measured by STFP evident only at 30 days post-MPTP. In addition, mice had a change in fear extinction at both 7 days and 30 days post-MPTP. In contrast, there were no significant changes in anxiety (measured by the hole-board and light–dark preference tests), or depression (measured by the sucrose preference and tail-suspension tests). Dopamine and serotonin levels in brain homogenates were depleted in the striatum, frontal cortex, and amygdala.

Dopaminergic neurotransmission within the basal ganglia has been implicated in cognitive processes, and specifically, in associative learning (Alcaro et al., 2007). However, only a few studies have examined memory function after basal ganglia injury in rodent models. A previous study reported that MPTP-lesioned CD-1 mice had impaired social memory and recognition behavior (Dluzen and Kreutzberg, 1993). In our study, the STFP test revealed impaired associative memory in mice only at 30 days post-MPTP-lesion compared to saline-treated mice. At this time point, there was still an 86% depletion of dopamine and a 60% depletion of serotonin within the frontal cortex. This is consistent with other studies using 6-OHDA-lesioning in rats where performance in the STFP test depends on an intact frontal cortex (Ross et al., 2005) and basal forebrain (Berger-Sweeney et al., 2000). Taken together, these data support the importance of the frontal cortex and the role of dopamine and serotonin in the associative learning processes.

The fear conditioning response is mediated by the basolateral amygdala, hippocampus, medial prefrontal cortex, and nucleus accumbens (Davis and Whalen, 2001; Helmstetter, 1992; LeDoux, 2000; Maren and Quirk, 2004). The extinction of conditioned fear is a progressive decrease of the fear response generated by repeated presentation of the conditioned stimulus (tone) without any unconditioned stimulus (foot-shock). Changes in the extinction of conditioned fear can be influenced by either glutamate or dopamine neurotransmission particularly in the frontal cortex and amygdala (Falls et al., 1992; Guarraci et al., 1999; Ledgerwood et al., 2003; Walker et al., 2002). For example, dopamine D1 and D2 receptor antagonists targeting the amygdala can lead to potentiated extinction of fear response (Greba et al., 2001; Greba and Kokkinidis, 2000; Nader and LeDoux, 1999; Ponnusamy et al., 2005). In our study, MPTP lesioning had no effect on the acquisition of the fear response; instead we observed increased fear extinction at both early and late time points after MPTP lesioning. Interestingly, a recent study in patients with PD reported decrease of the startle response to aversive stimuli and this behavior was linked to dopamine dysfunction in the amygdala and frontal cortex (Bowers et al., 2006). Although dopamine loss in the amygdala did not reach statistical significance in our MPTP-lesioned mice, altered cortical input to the amygdala could be responsible for observed behavioral responses. It is possible that significant loss of dopamine in the frontal cortex in mice at 7 and 30 days post-MPTP could be involved in this process. It is know that GABAergic interneurons surrounding the basolateral amygdala receive cortical and mesolimbic dopaminergic input (Marowsky et al., 2005). Modulation of dopamine gate in these neurons may modulate stress-induced behavioral responses (Bowers et al., 2006; Marowsky et al., 2005). Dopamine loss in the frontal cortex, which we observed in MPTP-lesioned mice, could cause disinhibition of these interneurons and block the output of the amygdala in response to aversive stimuli, thus preventing the normal fear induced freezing response.

Dopamine, serotonin, and other neurotransmitter system perturbations are involved in anxiety disorders and may account for the clinical anxiety seen in more than 40% of patients with PD (Walsh and Bennett, 2001; Wood and Toth, 2001). In our study, using light–dark exploration and the hole-board tests we found no increase in anxiety at either 7 or 30 days post-MPTP lesioning. These results are in agreement with others who also showed no difference in anxiety using the light–dark preference test 7 days after MPTP lesioning in the mouse (Rousselet et al., 2003). Depression is the most common co-morbid disorder affecting up to 45% of patients with PD (Slaughter et al., 2001). We examined depression in our model using both the tail suspension and sucrose preference tests, since both may be influenced by serotonin dysfunction (Jones and Lucki, 2005; Lira et al., 2003; Mayorga et al., 2001; Steru et al., 1985). We did not observe increased depression in MPTP-lesioned mice using these tests. Interestingly, a recent study using the bilateral 6-OHDA-lesioned rat reported an increase in depression-like behavior that was measured using the forced swim test, with no changes in serotonin levels (Branchi et al., 2008). It is possible that the neurotoxicant lesioning paradigm, time after lesion, and species used could underlie differences in observed behaviors in these two animal models.

Lesioning of the dopaminergic system, using 6-OHDA or MPTP, also leads to perturbations of the serotonergic system. However, the extent and nature of these perturbations depends on a number of factors including animal age, toxin used, lesioning regimen, and lesion severity. For example, rats and mice lesioned with 6-OHDA in early postnatal life develop serotonergic hyper-innervation within the striatum and frontal cortex and elevated levels of serotonin (Avale et al., 2004; Berger et al., 1985; Snyder et al., 1986; Yamazoe et al., 2001). This is in contrast to lesioning in adults where serotonin levels remain unchanged (Branchi et al., 2008; Snyder et al., 1986; Stachowiak et al., 1986). On the other hand, MPTP lesioning in adult animals causes a significant decrease of striatal and extra-striatal serotonin levels. For example, in the nonhuman primate, MPTP lesioning causes decreased levels of serotonin in multiple brain

regions including the caudate nucleus, putamen, nucleus accumbens, hypothalamus, and cerebral cortex (Frechilla et al., 2001; Perez-Otano et al., 1991; Pifl et al., 1991; Russ et al., 1991). In our studies, we found significant depletion of serotonin following MPTP lesioning in the mouse, a similar effect reported by others (Rousselet et al., 2003) but not all (Sedelis et al., 2000). Despite serotonin depletion in brain regions important for affective behavior, our MPTP-lesioned mouse did not show significant changes in anxiety and depression. The lack of behavioral effect could be explained by (i) the level of serotonin depletion may not be severe enough to manifest elevated anxiety, or (ii) the affected neurotransmitter systems may compensate to overcome perturbation. The serotonergic system following MPTP lesioning may compensate in an analogous fashion to that of the dopaminergic system where studies in our lab have shown recovery of dopamine function due to increased evoked release of dopamine and altered dopamine receptor expression (Petzinger et al., 2007). On the other hand, studies in PD patients have shown neuronal loss in the dorsal raphe nucleus which could cause mood disorders (Agid and Blin, 1987; Paulus and Jellinger, 1991; Scatton et al., 1983). However, these abnormalities in humans are thought to develop at later stages of the disease, following dysfunction of the dopaminergic system (Dauer and Przedborski, 2003). With this in mind, there is a possibility that behavioral changes in our acute MPTP-lesioned mouse may develop at later time points after treatment. Future studies could be designed to examine different lesioning regimens (acute versus chronic), different time points after lesioning (early versus late), and could include pharmacological challenges to the serotonergic system to reveal dysfunction in this neurotransmitter system. In addition, our initial analysis of neurotransmitter changes involved tissue sections such as the ventral striatum that did not delineate potentially interesting anatomical sites such as the core and shell of the nucleus accumbens. Future studies using higher-resolution techniques such as molecular imaging can be designed to examine altered innervation and synaptogenesis within these regions thought to influence non-motor behaviors.

The MPTP-lesioned mouse is commonly used as an animal model of PD. Specific motor functions such as skilled forepaw use, balance, and coordination have been consistently reported to be impaired following MPTP lesioning (Meredith and Kang, 2006; Meredith et al., 2008; Rozas et al., 1998; Sedelis et al., 2000; Tillerson et al., 2002; Tillerson and Miller, 2003). The behavioral tests used in our study do not rely on any of these specific motor skills. For example, saline control and MPTP-lesioned mice showed similar levels of ambulatory activity in the hole-board and light–dark preference tests. We therefore conclude that MPTP-lesioned mice in the present study did not exhibit obvious impairment of spontaneous locomotor activity that could influence their performance in affective behavior tests used in these studies.

In conclusion, our data showed impairment in associative memory at 30 days and increased fear extinction at both 7 and 30 days post-MPTP lesioning, but no significant increase in depression or anxiety. Impairments in memory and fear conditioning were accompanied by severe depletion in dopamine and serotonin levels in the amygdala, frontal cortex, and striatum. It is possible that the emergence of depression and anxiety in this mouse model depends upon greater serotonin loss in critical brain regions. The findings from this study suggest that mood disorders in patients with PD may not develop before extensive damage in multiple brain regions occurs.

Acknowledgments

The authors would like to acknowledge the generous support of Team Parkinson LA, The Parkinson Alliance, and the Whittier PD Education Group. This study was funded by NINDS RO1 NS44327-1, NIDCD K18 DC009125, and U.S. Army NETRP (Grant #W81XWH-04-1-0444). We are thankful to Friends of the USC Parkinson's Disease Research Group including George and MaryLou Boone and Walter and Susan Doniger for their generous support. Authors would like to thank Susan Geffen for help with statistical analysis. We would also like to thank Dr. Zhou Wang for help with fear conditioning data analysis.

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"Altered AMPA-Receptor Expression with Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Lesioned Mouse Model of Basal Ganglia Injury"

Journal:	*Journal of Neuroscience Research*
Manuscript ID:	jnr-2009-Mar-2868.R2
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	22-Jun-2009
Complete List of Authors:	VanLeeuwen, Jon-Eric; University of Southern California, Neurology Petzinger, Giselle; University of Southern California, Neurology Walsh, John; University of Southen California, Andrus Gerontology Center Akopian, Garnik; University of Southen California, Andrus Gerontology Center Vuckovic, Marta; University of Southern California, Neurology Jakowec, Michael; USC, Neurology
Keywords:	Parkinson's disease, MPTP, Glutamate receptors, Exercise, AMPA receptors

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Altered AMPA-Receptor Expression with Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Lesioned Mouse Model of Basal Ganglia Injury.

Running Title: AMPA-receptor changes with exercise

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Grant Information: The authors would like to acknowledge the generous support of the Parkinson's Disease Foundation, Team Parkinson LA, Parkinson Alliance, NINDS RO1 NS44327-1, NIA RO1 AG 21937, and U.S. Army NETRP (Grant # W81XWH-04-1-0444).

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ABSTRACT

Dopamine depletion leads to impaired motor performance and increased glutamatergic-mediated hyperexcitability of medium spiny neurons in the basal ganglia. Intensive treadmill exercise improves motor performance in both saline treated and the MPTP-lesioned mouse model of Parkinson's disease. In the present study we investigated the effect of high intensity treadmill exercise on changes in alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) subunit expression since these receptor channels confer the majority of fast excitatory neurotransmission in the brain and their subunit composition provides a key mechanism for regulating synaptic strength and synaptic neuroplasticity, and are important in modulating glutamatergic neurotransmission. Within the dorsolateral striatum of MPTP-lesioned mice, treadmill exercise increased GluR2 subunit expression with no significant effect on GluR1. Furthermore, neurophysiological studies demonstrated a reduction in the size of excitatory post-synaptic currents (EPSCs) in striatal medium spiny neurons (as determined by the input-output relationship), reduced amplitude of spontaneous EPSCs, and a loss of polyamine sensitive inward rectification, all supportive of an increase in heteromeric AMPAR channels containing the GluR2 subunit. Phosphorylation of GluR2 at Serine-880 in both saline and MPTP mice suggests that exercise may also influence AMPAR trafficking and thus synaptic strength within the striatum. Finally, treadmill exercise also altered flip isoforms of GluR2 and GluR1 mRNA transcripts. These findings suggest a role for AMPARs in mediating the beneficial effects of exercise and support that adaptive changes in GluR2 subunit expression may be important in modulating experience-dependent neuroplasticity of the injured basal ganglia.

Keywords: Parkinson's disease, glutamate, dopamine, experience-dependent neuroplasticity, electrophysiology.

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INTRODUCTION

At all stages of life, the environment profoundly influences the vertebrate brain. In the adult animal, studies have demonstrated that experience in the form of exercise or an enriched environment can lead to changes in synaptic strength and neuronal excitability in regions of the CNS, such as the hippocampus and cortex (Dietrich et al. 2005; Kleim et al. 1996; Mora et al. 2007; Naka et al. 2005; Vasuta et al. 2007). However, experience-dependent plasticity at the level of the basal ganglia has not been thoroughly investigated. Motor control is mediated by the basal ganglia and is highly influenced by glutamatergic input from the cortex and thalamus. The ability of the glutamatergic system to modulate neuronal excitability, through synaptic modification, combined with its role in motor learning, suggests that this system may contribute to the dynamic changes that take place in the basal ganglia in response to experience, including exercise.

An important target for glutamate within the basal ganglia is the alpha-amino-3-hydroxy-5-methyl-4-isoazoleproprionic acid receptor (AMPAR) subtype of glutamate receptors. The AMPAR is responsible for the majority of fast excitatory neurotransmission within the central nervous system (CNS) and is a heteromeric tetramer consisting of subunits GluR1 through 4 (Greger et al. 2007). The most abundant AMPAR subunits in the striatum are GluR1 and GluR2 (Deng et al. 2007; Wang et al. 2004). Subunit composition and phosphorylation states of AMPARs regulate synaptic connectivity and strength as reflected through processes including long-term potentiation (LTP) and long-term depression (LTD), which are directly influenced by behavioral experience (Ehrlich and Malinow 2004; McCormack et al. 2006; Rao and Finkbeiner 2007; Takahashi et al. 2003). Although AMPARs are abundantly expressed within striatal medium spiny neurons (MSNs), their precise role in experience-dependent plasticity within the basal ganglia is poorly understood.

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Dopamine depletion in the basal ganglia leads to impaired motor function accompanied with MSN hyperexcitability. *In vitro* studies have demonstrated increased glutamatergic neurotransmission, through an increase spontaneous evoked post-synaptic current (sEPSC), amplitude and frequency, which may be due to pre- and post-synaptic mechanisms at corticostriatal terminals (Calabresi et al. 1993; Cormier and Kelly 1996; Tang et al. 2001; Tseng et al. 2001). Using the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of dopamine depletion, we have previously shown that intensive treadmill exercise improves motor performance and normalizes *pre-synaptic* glutamate immunolabeling within striatal terminals, suggesting a potential role for exercise in attenuating glutamatergic overactivity (Fisher et al. 2004; Petzinger et al. 2007). To further elucidate exercise-induced changes in post-synaptic glutamatergic properties in the dopamine depleted state and to define the role of AMPARs in experience-dependent plasticity of the basal ganglia, we examined changes in the pattern of expression of the AMPAR subunits GluR1 and GluR2, two subunits involved in synaptic plasticity and learning (Kopec et al. 2007; Lee et al. 2003; McCormack et al. 2006). Our findings indicate that improved motor performance observed after intensive treadmill exercise is accompanied by an increase in both GluR2 subunit protein and transcript in the dorsolateral striatum of MPTP mice and an increase in phosphorylation of GluR2 at Ser880 in both saline and MPTP mice. Parallel with these findings sEPSC amplitude was increased in MPTP mice, while no changes in sEPSC amplitude or frequency were observed between MPTP mice after exercise and saline animals, Taken together, our studies suggest that exercise-induced attenuation of glutamatergic-mediated hyperexcitability, as measured through sEPSC amplitude, may be due to post-synaptic mechanisms that include alterations in GluR2-containing AMPARs. In addition, our findings support the role of AMPARs in mediating the beneficial effects of exercise and suggest that changes in AMPAR subunit expression and phosphorylation states may be important in modulating experience-dependent plasticity of the injured basal ganglia, including Parkinson's disease.

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METHODS AND MATERIALS

Animals

Mice used for these studies were young adult (8 to 10 weeks old) male C57BL/6J mice supplied from Jackson Laboratory, Inc. (Bar Harbor, Maine). A total of 100 mice were used in these studies and the number for each procedure is indicated in each relevant methods section. There were 4 treatment groups including: (i) saline (n=22), (ii) saline plus exercise (n=22), (iii) MPTP (n=29), and (iv) MPTP plus exercise (n=27). Animals were housed 5 to a cage and acclimated to a 12-hour shift in light/dark cycle so that the exercise occurred during the animals normal wake period. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and approved by the University of Southern California Institutional Animal Care and Use Committee (IACUC).

MPTP-Lesioning

MPTP (Sigma, Inc, St. Louis, MO) was administered in a series of 4 intraperitoneal injections of 20 mg/kg (free-base) at 2-hour intervals for a total of 80 mg/kg. This regimen leads to approximately 60% loss of nigrostriatal neurons as determined by unbiased stereological techniques for both TH staining and Nissl substance and an 80-90% depletion of striatal dopamine levels (Jackson-Lewis et al. 1995; Jakowec et al. 2004). Nigrostriatal cell loss is complete by day 3 after MPTP administration as measured by counting remaining nigrostriatal tyrosine hydroxylase immuno-reactive cells and reduced silver staining for degenerating neurons (Jackson-Lewis et al. 1995; Jakowec et al. 2004).

Treadmill Exercise

One week before the start of the treadmill exercise paradigm, mice that could maintain a forward position on the 45-cm treadmill belt for 5 minutes at 5.0 m/min were randomly assigned to the 4 groups to insure that all animals performed similarly on the treadmill task prior to MPTP

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lesioning. The treadmill used in these studies was a 6-lane Model EXER-6M Treadmill manufactured by Columbus Instruments (Columbus, Ohio). A non-noxious stimulus (metal beaded curtain) was used as a tactile incentive to prevent animals from drifting off the treadmill. As a result, shock-plate incentive was not used and stress related to the activity was minimized. Treadmill exercise was initiated 5 days following saline or MPTP injections, when MPTP-induced cell death is complete. Mice from each of the two exercise groups (saline plus exercise and MPTP plus exercise) were run simultaneously in different lanes. Exercise duration was incrementally increased starting with 30 minutes on day 1 to reach a goal duration of 2 sessions of 30 minutes each (for a total of 60 minutes), 5 days/week (with a 5 minute warm-up period) for a total of 28 days of exercise (corresponding to a final of 42 days after MPTP administration). To maintain comparable degrees of motor challenge for both exercise groups, treadmill speed and exercise duration for each group was increased when all mice within each group maintained a forward position on the treadmill, for 75% of the running period. To control for any non-exercise effects of treadmill running (handling, novel environment, noise, and vibration) non-exercised groups were placed on the top of the treadmill apparatus for a time period equivalent to exercise training (Fisher et al. 2004; Fukai et al. 2000; Kojda et al. 2001). At the end of the running period, all animals from the four groups (exercise and non-exercise, with and without MPTP) were run to compare running speed capability. Treadmill velocity was set at the same speed at which initial pre-exercise baseline running capability was determined (i.e., 10m/min). Maximum velocity was determined at which speed the mice as a group of 10 could maintain a forward position on the treadmill for 75% of a 5-min trial.

Tissue Collection

Brain tissue from all groups of mice was collected on the last day of exercise. Striatal brain tissue was also collected from a subset of animals from the saline and MPTP experimental groups at 10-days post-lesioning to determine the degree of dopamine depletion at an earlier MPTP time point. Mice were sacrificed by cervical dislocation for fresh tissues (for HPLC, qRT-

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PCR, and electrophysiology) or by pentobarbital overdose followed by transcardial perfusion with fixative (for immunohistochemistry). Striatal tissues for HPLC and qRT-PCR analysis were collected fresh en block corresponding to anatomical regions Bregma 1.20 to 0.60, with borders dorsal to the anterior commissure, ventral to the corpus callosum, medial to the lateral ventricle, and lateral 2.5 mm from midline and frozen until analysis. Immunohistochemistry and electrophysiology were performed on coronal sections corresponding to Bregma 1.30 to 0.00 Bregma.

HPLC Analysis of Dopamine and its Metabolites

Dopamine concentration, its metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), and turnover ratio (DOPAC + HVA)/dopamine] were determined according to an adaptation of Irwin et al (1992) of the method of Kilpatrick and colleagues (1986) (Irwin et al. 1992; Kilpatrick et al. 1986) and used in our previous reports (Petzinger et al. 2007).

Immunohistochemical Staining

All proteins were visualized using an immuno-peroxidase labeling method on fixed mouse tissue. Mice (n = 4 per treatment group) were administered pentobarbital (160 mg/kg, i.p.), perfused transcardially with cold saline followed by 4% paraformaldehyde/phosphate-buffered saline, pH 7.2 (called 4% PFA/PBS). After perfusion, brains were removed, immersion fixed in 4% PFA/PBS at 4ºC for 48 hrs, then cryoprotected in 20% sucrose until they sank, then quickly frozen in 2-methylbutane on dry ice, and stored at -80ºC. Tissue was cut into 25-µm-thick sections and placed in PBS, pH 7.2 for immediate use. Sections were rinsed in TBS three times, quenched in 10% methanol/ 10% H_20_2/ 50mM Tris, pH 7.2, blocked for one hour in 4% normal serum, and exposed to monoclonal or polyclonal primary antibody (concentration of 1:1000) for 24 hours at 4ºC. Polyclonal probes made in rabbit included GluR1, GluR1 phosphorylated at Serine845, GluR2 phosphorylated at Serine880 (Chemicon, Temecula CA.); monoclonal antibodies made in mouse included GluR2 (Antibodies Inc., Davis CA). Sections

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were then rinsed three times in TBS, incubated in secondary antibody (concentration 1:500) made against the species of the primary antibody for 1 h and then in avidin-biotin complex using the ABC elite kit; (Vector Inc., Burlingame, CA). Sections were visualized in 0.1% 3,3'-diaminobenzadine tetrahydrochloride and 0.1% H_20_2. Antibody specificity was validated by subjecting sections to the same experimental conditions but without the addition of primary and/or secondary antibody, After staining, sections were mounted on gelatin subbed slides, dried, cleared in xylenes and then cover-slipped.

Relative Quantification of Tissue Immunoreactivity

Semi-quantitative analysis was carried out to determine the relative immunoreactivity against GluR1 and GluR2 and their phosphorylated states with antibody specific probes within the dorsolateral striatum of tissues form all treatment groups. Both (i) the total number of immunoreactive cell bodies and (ii) the intensity of neuropil immunoreactivity were determined. We used a sampling technique with distinct boundaries selected to collect representative sections though the dorsolateral striatum, a region important for motor control. Tissues were sectioned at 25-micron thickness starting at bregma 1.18 (where the anterior commissure juxtaposes below the dorsal tip of the lateral ventricle) to bregma 0.26 where the anterior commissure begins to bridge the two hemispheres (Paxinos and Franklin 2001). Tissues were collected in a 6-well tissue culture dish placing tissue sections in alternating wells in a permutation of 6; thus each well contains every sixth section and thus avoids analysis of adjacent sections. Each analysis consisted of 6 sections per animal from 4 different animals. Digitized images were captured in TIFF format through an Olympus BX-51 light microscope interfaced with a Retiga 3000 camera and the computer assisted image analysis program Bioquant Nova Prime (Bioquant Imaging, Nashville, TN). The number of immunoreactive cell bodies within the dorsolateral striatum was determined at 40X objective magnification targeting a region of interest (ROI) consisting of a 0.15 square mm field bound laterally and ventrally by the corpus callosum and laterally to a 45 degree line joining the apex of the corpus callosum (1

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mm medial and 1.2 mm from the most dorsal edge of the cortex) to the ventral and lateral edge of the corpus callosum (2.5 mm medial and 3.25 mm from the most dorsal edge of the cortex). A threshold based on three-color channels, which uses 24-bit color discrimination, was created to automatically differentiate cell bodies from background or other artifacts. Using the intensity established by setting the threshold, cell bodies were manually selected based on size (greater than 15 microns), morphology (appearance of dendritic arbor, large soma, and intact nucleus), location (within the perimeter of the dorsolateral striatum), and automatically counted. The relative optical density (OD) of the neuropil within immunoreactive tissue sections was determined at high 100X objective magnification. A typical ROI (.023 square mm) was selected that did not include stained cell bodies or artifacts. Measurements produced values ranging from 0 (black) to 255 (white). Each value was subtracted from 255 such that a larger value corresponded to greater immunoreactivity. To control for non-specific staining, measurements from the corpus callosum of the same tissue section were used as background and subtracted from total neuropil staining. Multiple sections from each of the different treatment groups were handled in identical staining conditions concurrently to ensure that any differences in staining intensity were due to differences in antigen expression. For analysis, all treatment groups were normalized against tissue from saline treated mice.

Western Immunoblotting for GluR1 and GluR2 Protein

Western immunoblotting was used to determine the relative expression of GluR1 and GluR2 proteins within the dorsolateral striatum. A total of 6 mice were used from each group. Following decapitation, synaptoneurosome were prepared as previously described (Johnson et al. 1997), with slight modifications (Banko et al. 2004; Villasana et al. 2006). Brain tissue was homogenized with a Teflon-homogenizer (4 strokes at 1000 rpm) in buffer (1/10 wt/vol) containing 0.35M sucrose pH 7.4, 10mM HEPES, 1mM EDTA, 0.25mM dithiothreitol, 30 U/ml RNAse inhibitor and a protease inhibitor cocktail (Calbiochem, Inc). Cell debris and nuclei were removed by centrifugation at 1000g for 10 min at 4°C yielding pellet P1 and supernatant S1.

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The S1 fraction was passed through a series of 4 nylon mesh screens with decreasing pore size finishing with passage through a 5-micron screen. The final filtrate was resuspended in 3 volumes of buffer and centrifuged at 2000g for 15 minutes at 4°C. Protein concentration was determined by the BCA method (Pierce, Inc). Equal amounts of protein (20 µg) were separated by the method of Laemmli (Laemmli 1970). Proteins were transferred to nitrocellulose filters by electroblotting in Towbin buffer (Towbin et al. 1979). Antibodies for western immunoblotting were the same as used for immunohistochemistry except an antibody against alpha-tubulin (Millipore, Inc., Temecula, CA) was used to determine equal loading of total protein per gel lane. The immunoblotting technique was previously described (Jakowec et al. 2001; Jakowec et al. 1995a; Jakowec et al. 1998) with modifications using the Li-cor Odyssey for Near Infra Red Fluorescent Western Blotting (Lincoln, NE). Filters were blocked in Rockland Blocking Solution (Gilbertsville, PA), then exposed to primary antibody (1:1000) in the same Blocking Solution, exposed to secondary antibody, and visualized using the Li-cor Odyssey. The intensity of bands was determined using the image analysis program (Li-cor, Inc., Lincoln, NE) and expressed as relative optical density.

In Situ Hybridization Histochemistry for GluR1 and GluR2 mRNA Transcripts

Brains for *in situ* hybridization were quickly removed at the end of the exercise regimen and frozen in isopentane on dry ice and tissues processed as previously described (Jakowec et al. 2004; Jakowec et al. 1995b). Selected slides were dipped in NTB-2 (Kodak) photographic emulsion, developed in D-19 developer and counter stained with cresyl violet. To minimize potential sources of variation between different experiments, slides to be compared were processed in the same experiment using identical hybridization buffers, probe concentration, probe preparation, wash regimen, and emulsion exposure. Images of dorsolateral cells were captured using an Olympus BX-51 microscope and the computerized image analysis program Bioquant (Bioquant Imaging, Nashville, TN) was used to determine the number of emulsion grains above individual neurons that were counterstained for Nissl substance. Results were

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reported as number of grains per cell area. A total of at least 15 dorsolateral striatum cells were counted in each of 4 different animals per group.

Quantitative Real-time PCR

Total RNA was isolated from n= 4 mice per group using the RNeasy Lipid Tissue Mini Kit and RNeasy Mini Spin Column from Qiagen (Valencia, CA) following the manufacturers instructions. RNA was quantified by measuring absorbency at 260 nm. Reverse-transcription to generate first strand complementary DNA (cDNA) was achieved by mixing 5µg of total RNA with 2µL Oligo $(dT)_{20}$ Primer, 1µL of Omniscript Reverse Transcriptase, 2µL of dNTP mix, 2µL of 10X Buffer, and 1µL of RNase inhibitor (Omniscript RT Kit, Valencia, CA) and incubated for 60 min. at 37°C, followed by 5 min at 93°C. The reverse-transcribed cDNA was diluted with RNase-free water to a final of 1:200 and used as the template for analysis. Gene expression of AMPA receptor (AMPA-R) subunits within the striatum were determined with mouse primer sets specific for GluR1 and GluR2 flip and flop splice variants; (1), GluR1 (Forward) 5'-ACACCATGAAAGTGGGAGGTAACT-3'; (2) GluR1-flip (Reverse) 5'-ACTGGTCTTGTCCTTACTTCCGGA-3'; (3) GluR1-flop (Reverse) 5'-ACTGGTCTTGTCCTTGGAGTCACC-3'; (4) Glu2 (Forward) 5'-ACACCATGAAAGTGGGCGGCAACC-3'; (5) Glur2-flip (Reverse) 5'-ACTGGTCTTTTCCTTACTTCCCGA-3'; (6) GluR2-flop (Reverse)5'-ACTGGTCTTTTCCTTGGAATCACC-3'. Quantitative real-time PCR (qRT-PCR) analysis was performed with the Mastercycler ep Realplex^2 PCR system (Eppendorf, Inc) using 20x SYBR Solution RealMasterMix (Eppendorf, Inc). In the qRT-PCR protocol, samples were subjected to denaturation at 95°C for 2 min, followed by 45 cycles of amplification and quantification at 95°C for 15 s and 58°C for 15 s, respectively, with one cycle of the finishing program (68°C for 20 s). The qRT-PCR efficiency was verified by melting curve analysis. To accurately assess the amount of RNA expression, each sample was normalized to the housekeeping gene encoding

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glyceraldehyde-3-phosphate (GAPDH) using the primer sequence, (Forward) 5'-TGCACCACCAACTGCTTA G-3'; (Reverse) 5'-GGATGCAGGGATGATGTTGTTC-3'.

Electrophysiological Studies

Mice from all groups were anesthetized in a dessicator containing halothane vapors, killed by decapitation, and brains were removed. Tissue was blocked in cold low-sodium sucrose-substituted saline (90 mM saline with 105 mM sucrose) and striatal coronal sections were cut at 350-µm thickness in ice-cold low-sodium sucrose-substituted saline using a Vibratome-1000 (Vibratome Co., St Louis, MO). Slices were stored in artificial cerebral spinal fluid (aCSF consisting of 124 mM NaCl, 1.3 mM $MgSO_4$, 3 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.4 mM $CaCl_2$, and 10 mM glucose) at room temperature (23^{o}C) for at least one hour prior to recording. All solutions were continuously oxygenated with 95% O_2 and 5% CO_2. Slices were then transferred to a submerged brain slice-recording chamber perfused with oxygenated aCSF kept at a recording temperature of 32^{o}C as outlined in (Akopian and Walsh 2007). The pH of all oxygenated solutions was 7.4. Picrotoxin (50 µM) was used to block gamma-amino butyric acid-A ($GABA_A$) receptor mediated inhibition in an attempt isolate excitatory synaptic events.

Whole cell voltage clamp and electrical stimulation methods were used to examine corticostriatal synaptic input. Voltage clamp was chosen as the recording method to reduce possible activation of postsynaptic conductance's, which can contribute to changes in synaptic strength under current clamp conditions (Akopian and Walsh 2002). Whole cell recordings were obtained from striatal medium spiny neurons identified visually using a fixed stage microscope and water immersion lenses (Zeiss Axioscope, Germany). Intracellular electrodes contained 0.5% biocytin (Sigma-Aldrich, Inc.) in some experiments to verify the cell type based on morphology. Patch electrodes were pulled with a Flaming-Brown P-87 pipette puller (Sutter Instrument, Novato, CA) from thin-wall borosilicate capillary glass having a 1.5 mm outer diameter (o.d.) (WPI, Sarasota, FL). The electrodes had resistances ranging between 4 and 6

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MΩ when filled with the pipette solution. The pipette (internal) solution was composed of (mM): Cs gluconate 130, CsCl 10, EGTA 5, $MgCl_2$ 2, HEPES 10, QX-314 5, ATP-Mg 2, GTP-Na 0.25, pH 7.25, 285 mOsm. Spermine (100 µM) (Sigma-Aldrich Inc.) was included to provide polyamine modulation of GluR2 lacking AMPA receptors. The liquid junction potential between the pipette and aCSF were estimated as 15 mV. Cells were held at different holding potentials during the time course of experiments as needed, taking into account this liquid junction potential. Series resistance (Rs) was monitored throughout the experiment by measuring the instantaneous current response to 5 ms hyperpolarizing (-5 mV) pulses delivered before synaptic stimulation and was not compensated. A gravity-fed array of inflow tubes of ~100 µm inner diameter and an outflow tube attached to a vacuum reservoir provided solution flow. The ground electrode consisted of a salt bridge constructed from glass electrode filled with agar. Passive membrane properties of the cells in slices were determined in voltage clampmode with the "Membrane Test" option of the "Clampex 9" software by using 10 mV depolarizing step voltage command from a holding potential of 70 mV.

Changes in the amplitude and frequency of spontaneous excitatory postsynaptic currents (sEPSCs) were sampled for 2 minutes. Neurons were voltage clamped at -70 volts during recording. The membrane currents were filtered at 1 kHz and digitized at 5 kHz. sEPSCs were analyzed off-line using the threshold detection option found in Clampfit 9 (Molecular Devices, Sunnyvale, CA). The threshold amplitude for the detection of an event was set at above 5 pA. Cumulative frequency histograms were generated for sEPSC amplitude and inter-event interval. The bin size for sEPSC amplitude was 2 pA and the bin size for sEPSC inter-event interval was 100 msec.

Stimulus-evoked excitatory postsynaptic currents (EPSCs) in the lateral portion of the dorsal striatum were generated using stimulation electrodes filled with aCSF and positioned 100-500 micrometers from the recording electrode at the border between striatum and the overlying corpus callosum. Rectangular current pulses of 0.1 ms duration were applied to the stimulation electrodes relative to a reference electrode placed in the recording chamber using

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Stimulus Isolation Unit A365 (WPI, Sarasota FL) triggered by digital output of Digidata 1320. Input (stimulation intensity) – output (synaptic response) relationships were determined for corticostriatal synapses by applying a standard ascending sequence of stimulus intensities and recording excitatory postsynaptic currents (EPSCs). Neurons were voltage-clamped at -80 mV during periods of stimulation. The slope of the input output relationship was determined for each cell and compared using one-way repeated ANOVA. To estimate the paired pulse ratio (PPR) the five paired pulse synaptic stimulations with the inter-stimulus interval of 50 ms at holding potential of -70 mV were delivered through the stimulating electrode with 20 sec intervals. The intensity of synaptic stimulation was set at about 50% of maximum responses obtained from I/O curve. The five traces were averaged and PPR was expressed as percentage of the ratio of the second pulse to the first one in the series.

The rectification index (RI) was determined as the slope of the synaptic current-voltage relationship (I-V) curve at positive potentials (0 to +60 mV) divided by the slope of synaptic I-V curve at negative potentials (-80 to 0 mV) (Liu and Cull-Candy 2005; Shin et al. 2007). Synaptic current-voltage relationships (I-Vs) were obtained by generating synaptic currents with electrical stimulation of cortical afferents every 20 sec at different holding potentials ranging from –80 mV to +60 mV with increments of 20 mV. The stimuli were delivered 5 sec after the stepped change in the holding potential. All RI experiments were performed in slices bathed in picrotoxin to block $GABA_A$ receptor mediated responses and AP-5 (50 μM) to block NMDA receptor mediated responses.

Statistical Analysis of Data

Statistical analyses were performed using SPSS version 14.0 for Windows (SPSS, Inc., Chicago, IL) or GraphPad Prism 4 (San Diego, CA). Differences between groups in behavioral tests were analyzed using repeated-measures analysis of variance (ANOVA) with the between-subjects factors being lesion (saline or MPTP) and the within subject factor being time. For HPCL analysis of 10 days post-lesioning animals, an unpaired Students t-test was performed for

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comparisons between MPTP versus saline treated mice. For HPLC analysis, western immunoblotting, in situ hybridization histochemistry, qRT-PCR, and immunohistochemistry staining of the 42 days post-lesioning animals a two-way ANOVA was performed to compare the different groups and to examine for significant interactions. Post-hoc contrasts with Bonferroni correction were performed to determine the locus of any significant differences. For physiological studies, comparisons between MPTP, MPTP plus exercise, and saline mice for the slope of the input output relationship and the RI were performed using one-way ANOVA. Between-group comparison of sEPSC amplitudes and interevent intervals was performed using repeated measures ANOVA across all bins. For all analyses a significance level of $p < 0.05$ was used.

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RESULTS

Intensive Treadmill Training Leads To Improved Running Velocity in MPTP Mice

Animals from both the MPTP and saline groups were able to complete the treadmill exercise regimen. The time course of improvement in running velocity of the saline plus exercise and MPTP plus exercise groups over the 6 weeks (28 days) of treadmill running is shown in Figure 1. In the first week of treadmill running, saline plus exercise mice started at a velocity of 14 ± 1.4 m/min, which increased to 22.6 ± 0.3 m/min by the final week. The MPTP plus exercise group had a running velocity of 9.2 ± 1.1 m/min during the first week that increased to 20.5 ± 0.7 m/min in the last week. As we have previously reported, there was a significant difference in velocity at weeks 1-4 between the saline plus exercise and MPTP plus exercise groups ($P < 0.05$). This difference was eliminated with further training and completion of the treadmill running regimen. At the end of the six week exercise period, there was no spontaneous improvement in treadmill running performance in the MPTP mice (7.5 ± 0.2 m/min) in the absence of treadmill training compared with MPTP plus exercise mice ($P < 0.05$) (Fisher et al. 2004; Petzinger et al. 2007).

Exercise Does Not Alter Striatal Dopamine Levels in MPTP Mice

In order to examine the effects of exercise on striatal dopamine levels, HPLC analysis for dopamine, its metabolites, and turnover ratio was conducted on all groups at the termination of the treadmill exercise regimen (42 days post MPTP administration) (Table 1). Additionally, a subset of animals was examined at 10-days post-MPTP administration to establish the degree of dopamine depletion at the commencement of the treadmill exercise. Ten days after MPTP lesioning mice showed significantly lower levels of striatal dopamine (48.0 ± 8.4 ng dopamine/mg protein) compared to the saline group (269.5 ± 24.9 ng dopamine/mg protein) ($p < 0.05$), which represented an 83% depletion. Analysis of dopamine turnover ratio showed a

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significant elevation in the MPTP group (turnover ratio = 2.3), compared to the saline group (turnover ratio = 0.3) ($p < 0.05$). At the completion of the 28 days of treadmill running (42 days post-MPTP lesioning), dopamine levels remained significantly depleted in MPTP mice (68% depletion) compared with saline group ($F(3,16) = 229.3$, $p < 0.0001$). As previously reported there was no significant difference in striatal dopamine levels comparing MPTP plus exercise with MPTP mice (Petzinger et al. 2007). There was a significant effect of exercise on the saline treated group, where saline plus exercise mice had a higher level of striatal dopamine compared to saline mice ($F(3,16) = 7.78$, $p < 0.05$).

Exercise Increases Expression of GluR2 Protein and its Phosphorylated Form at Serine 880

Previous studies using immunoelectron microscopy had shown that exercise normalizes the density of pre-synaptic nerve terminal glutamate immunolabeling (Fisher et al. 2004). To determine whether exercise induces post-synaptic changes in glutamatergic signaling, we investigated changes in the protein expression of AMPAR subunits in MPTP mice after 28 days of intensive treadmill running using immunohistochemistry and western immunoblotting of tissue from the dorsolateral striatum. Antibodies specific for GluR2 or its phosphorylation at serine 880 were used to independently assess GluR2 and its phosphorylated form. Immunoreactivity of GluR2 subunit expression using a pan-specific antibody showed that the total number of immunoreactive-positive cells within the dorsolateral striatum significantly increased with exercise ($F(3,21) = 21.6$; $p = 0.016$) and that this exercise effect was most significant in the MPTP group ($F(3,21) = 47.04$; $p = 0.001$) (Figure 2). Analysis of GluR2-immunoreactivity within the neuropil of the dorsolateral striatum, however, showed no significant differences in relative optical density between the exercise and non-exercise groups ($F(3,21) = 0.178$; $p = 0.684$), or between the saline and MPTP-lesioned groups ($F(3,21) = 0.358$; $p = 0.566$). Changes in GluR2 protein expression were further analyzed using western immunoblotting of synaptoneurosomes from dorsolateral striatal tissue. Treadmill exercise led to a significant increase in GluR2 protein

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expression in MPTP mice F(3,21) = 4.4; p < 0.05) (Figure 3A). In addition to the effects on GluR2, we observed a significant exercise-induced increase in the number of immunoreactive-positive cells expressing phosphorylated GluR2-Ser880 in both MPTP and saline treated animals (F(3,21) = 19.22; p < 0.002) (Figure 4). There were no significant differences of phosphorylated GluR2-Ser880 immunoreactivity detected within the neuropil between any of the groups,

GluR1 Protein Expression is Unaltered by Exercise

Exercise had no significant effect on the total number of immunoreactive-positive cells expressing the GluR1 subunit (F(3,21) = 0.04; p = 0.846) (Figure 5). There was also no significant effect of MPTP on the total number of immunoreactive-positive cells in the dorsolateral striatum of any of the treatment groups (F (3,21) = 4.852; p = 0.0587). Analysis of GluR1-immunoreactivity within the neuropil showed no significant effect of either exercise (F(3,21) = 1.247; p = 0.297), or MPTP (F(3,21) = 1.599; p = 0.242). Similar to GluR1 expression, immunohistochemical staining for phosphorylated GuR1-Ser845 showed no significant effect of either exercise (F(3,21) = 1.493; p = 0.239), or MPTP (F (3,21) = 0.918; p = 0.352) on either the total number of immunoreactive-positive cells or neuropil immunoreactivity (exercise (F(3,21) = 0.999; p = 0.329), MPTP (F(3,21) = 1.0; p = 0.329)) (Figure 6). As observed with immunohistochemistry, there were no significant differences in the degree of GluR1 protein expression by western immunoblotting of synaptoneurosomes in any of the treatment groups (F3,21) = 1.5; p = 0.22) (Figure 3B).

MPTP Increases GluR2 mRNA Transcript Expression

In order to determine whether exercise or MPTP influences AMPARs at the level of transcription, we examined the relative expression of mRNA transcripts for GluR1 and GuR2 in the medium spiny neurons of the dorsolateral striatum, using sequence specific ribonucleotide probes and in situ hybridization histochemistry. Although there were no specific exercise effects,

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MPTP led to a significant increase in GluR2 transcript expression when compared to saline groups ($F(3,21) = 16.0$; $p < 0.01$) (Figure 7E to H). Analysis of GluR1 demonstrated a decreased expression of mRNA transcript in the saline plus exercise group compared to all other groups ($F(3,21) = 8.0$; $p < 0.005$) (Figure 7A to D).

Exercise Reduces GluR2 and GluR1 Flip Splice Variant Transcript Expression

AMPA-R subunits GluR1 and GluR2 are tightly regulated and undergo post-transcriptional modification leading to the generation of alternative splice variants termed flip and flop (Sommer et al. 1990). These splice variants result in different isoforms of AMPAR subunits that regulate heteromeric channel assembly and electrophysiological properties. For example, flip and flop splice variants modulate the kinetics of AMPAR desensitization by affecting glutamate binding to the receptor complex after channel closure (Quirk et al. 2004). Flip splice variants, in both GluR1 and GluR2, have larger synaptic currents and show less desensitization than flop (Koike et al. 2000; Mosbacher 1994; Partin et al. 1996; Sommer et al. 1990). To further examine potential exercise effects on AMPAR kinetics of glutamate binding, we used quantitative real-time polymerase chain reaction (qRT-PCR) to examine for differences in mRNA transcript expression encoding the flip and flop alternative splice variants for the GluR1 and GluR2 AMPAR subunits (Sommer et al. 1990). Analysis of mRNA splice variants showed that exercise led to a significant decrease in the expression of the flip isoform for both GluR2 ($F(3,12) = 35.90$; $p < 0.0003$), and GluR1 ($F(3,12) = 52.05$; $p < 0.0001$) in both MPTP and saline animals, with no significant effects on the flop isoform. MPTP caused a significant decrease in the expression of GluR2-flip ($F(3,12) = 22.47$; $p < 0.0015$) that was further decreased by exercise. MPTP also led to a significant increase in GluR2-flop (increase, $F(3,12) = 55.71$; $p < 0.0001$) and decrease in GluR1-flop (decrease, $F(3,12) = 103.3$; $p < 0.0001$) isoform (Figure 8).

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Treadmill Exercise Increases the Rectification Index for AMPAR-Mediated Synaptic Responses at Corticostriatal Synapses in MPTP mice

In order to further investigate the differential participation of GluR2 subunits in MPTP mice after exercise, and its relative dominance in synaptic AMPAR subunit expression, we used stimulus evoked current-voltage relationships to examine for the presence or absence of rectification. Specifically, AMPARs lacking GluR2 are permeable to Ca^{2+} and are characterized electrophysiologically by an inward-rectifying current-voltage relationship that is sensitive to polyamines (Bowie and Mayer 1995; Kamboj et al. 1995). Conversely, GluR2-containing channels do not bind polyamines and their current displays a linear current-voltage relationship (Pellegrini-Giampietro et al. 1997). Synaptic currents were evoked in dorsolateral striatal neurons by stimulating the overlying corpus callosum and the recording electrode contained the polyamine spermine (100 µM) Figure 9). Adding picrotoxin and AP-5 to the bathing media isolated AMPAR -mediated responses. Evoked synaptic responses were recorded at different holding potentials from -80 mV to +60 mV with 20 mV increments, and the RI between groups compared. A low RI is consistent with an inwardly rectifying current-voltage relationship, (e.g. AMPARs lacking GluR2 subunits), and a high RI is consistent with a linear current-voltage relationship (e.g. AMPARs containing GluR2 subunits). MPTP led to a significantly lower RI (0.64 ± 0.05 n=6) ($P < 0.03$) compared to all other groups, and treadmill exercise normalized the RI to that observed in saline mice. There was no significant difference between MPTP plus exercise (RI = 0.83 ± 0.05, n = 5) and saline treated mice (RI = 83 ± 0.07, n = 8). Taken together, these studies show that medium spiny neurons in the dorsolateral striatum of MPTP mice demonstrate a dominant synaptic expression of GluR2-lacking AMPARs and that treadmill exercise restores the expression of GluR2-containing AMPARs within the synapse to that observed in saline mice, by enhancing GluR2 protein expression.

Exercise reduces sEPSC amplitude in MPTP Mice

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We next examined for exercise-induced alterations in the electrophysiological properties of AMPARs and their pre-synaptic glutamatergic inputs through the recording of spontaneous excitatory post-synaptic currents, (sEPSCs) (Figure 10A). Spontaneous post-synaptic currents were reversibly blocked by the kainate/AMPAR antagonist CNQX (data not shown). In general, changes in sEPSC amplitude are interpreted to be due to post-synaptic mechanisms including changes in synaptic receptor number and/or channel conductance properties, while changes in sEPSC frequency are attributed to pre-synaptic mechanisms, including release properties of glutamate (Manabe et al. 1993; Maren 1993). We found the sEPSC amplitude was greater at excitatory striatal synapses from MPTP-treated mice compared to all other groups $F(1, 20)= 10.5$; $p<0.005$) and that 28 days of high-intensity treadmill exercise in MPTP-lesioned mice caused a significant reduction in sEPSC amplitude to levels seen in saline injected mice (Figure 10B). There were no significant differences observed in the frequency of sEPSCs amongst any of the treatment groups (Figure 10C). To further estimate the involvement of pre-synaptic mechanisms, specifically corticostriatal glutamatergic neurotransmission in the expression of synaptic modifications obtained by recording sEPSC in the experimental groups, we measured PPR, a sensitive indicator of neurotransmitter release properties. PPR in cortico-striatal slices from saline, saline plus exercise, MPTP and MPTP plus exercise mice were 87.71±3.35 (n=11), 91.91±3.77 (n=11), 80.19±2.19 (n=7) and 81.70±3.30 (n=10), respectively. No significant difference between groups was found ($p>0.05$), suggesting that MPTP-induced changes in sEPSC amplitude and synaptic hyperexcitability was not mediated by alterations in pre-synaptic glutamatergic release properties.

Treadmill Exercise Reduces the Input-Output Relationship at Corticostriatal Synapses in MPTP mice.

Given the role of AMPARs in synaptic plasticity, the effect of treadmill exercise on altering the strength of corticostriatal synapses was evaluated by delivering an identical sequence of stimuli (30-90 μA) to the corpus callosum in MPTP, MPTP plus exercise and saline

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mice (Figure 10A). All recordings were performed with cells held at a voltage of -80 mV to capture AMPAR mediated responses. A gradual increase of stimulation intensity was delivered to the corpus callosum overlying the dorsolateral striatum in order to produce a graded increase in the amplitude of corticostriatal EPSCs. The resulting input-output relationship for the synapses was used to evaluate the overall strength of corticostriatal connections, which could include changes in the overall release sites or changes in AMPAR properties. The slope of the EPSC amplitude versus the intensity of stimulation used to evoke the EPSC was determined. Exercise significantly reduced the slope of this relationship in MPTP mice ($p<0.03$) (Figure 10B). The slope of the input-output relationship for the MPTP group was 19.6 ± 2.7 (mean ± SEM, n=7) and the slope of the input-output relationship for the MPTP plus exercise group was 11.1 ± 1.7 (mean ± SEM, n=6) (Figure 10C). Interestingly, the slope of the input-output relationship for the saline group was 33.41 ± 3.72 (mean ± SEM, n=8) and was significantly higher than either of the MPTP groups ($p < 0.01$) (Figures 10B and 10C).

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Discussion

In the present study, we demonstrate that high intensity treadmill exercise leads to alterations in *post-synaptic* glutamatergic neurotransmission that are associated with changes in AMPAR subunit expression and its phosphorylation state within MSNs of the dorsolateral striatum, a region involved in motor control (Holschneider et al. 2007). The glutamatergic system plays an important role in motor learning. AMPARs, a subtype of the glutamate receptors, are responsible for the majority of fast excitatory neurotransmission in the CNS and mediate experience- and activity-dependent processes that alter synaptic strength (Engelman and MacDermott 2004; Takahashi et al. 2003; Wierenga et al. 2005). Both the number of receptors, their subunit composition and their trafficking through phosphorylation contribute significantly to the strength of excitatory synapses and are critically involved in synaptic plasticity, including LTD and LTP (Isaac et al. 2007; Jiang et al. 2006).

We found that intensive treadmill exercise leads to an increase in GluR2 transcript and protein in MPTP mice. Interestingly, an increase in GluR2 transcript, without an associated increase in protein, was observed in MPTP mice. In contrast to GluR2, we found no significant changes in the level of expression of total GluR1 protein after treadmill exercise. The lack of change in GluR1 protein expression, along with the increase in GluR2 expression in the MPTP plus exercise mice, suggests a shift in AMPAR composition, whereby GluR1-containing channels decrease and GluR2-containing channels increase. In order to further evaluate whether changes in GluR2 protein were accompanied with alterations in synaptic expression, we used current-voltage relationships in the presence of polyamine to examine for exercise-induced changes in the RI of MPTP mice. GluR2-containing channels do not bind polyamines and their current displays a linear current-voltage relationship (high RI), conversely GluR1-containing channels bind polyamine and display inward rectification (low RI) (Pellegrini-Giampietro et al. 1997). We observed the presence of inward-rectification (low RI) in MPTP mice, but observed a linear current-voltage relationship (high RI) after exercise. A high RI was

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also observed in saline animals. These findings provide electrophysiological evidence for a predominant expression of GluR1-containing AMPARs in MPTP mice, and GluR2-containing AMPARs in MPTP mice after exercise that may result from increased expression of the GluR2 subunit.

In our study, we also observed an exercise-induced increase in the phosphorylated state of GluR2 at Serine-880, which has been associated with increased trafficking of AMPARs (including GluR1-GluR2 and GluR2-GluR3 assemblies) away from the synaptic active zone and diminished synaptic strength. The dorsolateral striatum and cerebellum are both believed to be involved in motor learning and, interestingly, the predominant form of long-term plasticity expressed in these two structures is LTD (Mauk et al. 1998; Pisani et al. 2005). LTD expressed at the parallel fiber synapse with cerebellar Purkinje cells is mediated via phosphorylation of GluR2-Ser880 and subsequent internalization and retention of the receptor through its interaction with accessory proteins (Kakegawa and Yuzaki 2005; Korber et al. 2007; Lin and Huganir 2007; Seidenman et al. 2003; Song and Huganir 2002; Tomita et al. 2005). Findings from our study suggests that exercise based motor training alters synaptic strength and that this may be achieved, in part, by trafficking of AMPARs, which could lead to sustained changes in synaptic plasticity (Bredt and Nicoll 2003; Jiang et al. 2006; Malinow and Malenka 2002; Song and Huganir 2002).

The present study also demonstrates that exercise-induced improvement in motor performance is paralleled by a return of sEPSCs amplitude in striatal neurons to levels observed in saline mice. Specifically, MPTP mice displayed a potentiation of sEPSC amplitude in corticostriatal synapses of striatal MSNs, but no increase was observed in MPTP mice after intensive treadmill exercise. Spontaneous synaptic currents were blocked by CNQX but not by picrotoxin suggesting that they are mediated by AMPARs located on striatal neurons. Increased spontaneous firing of striatal neurons has been reported after dopamine depletion (Arbuthnott

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1974; Nisenbaum 1986; Orr et al. 1986; Schultz and Ungerstedt 1978; Tseng et al. 2001). In addition, *in vitro* evidence supports that corticostriatal transmission is increased in slices from 6-OHDA rats (Calabresi et al. 1993). Increased MSN excitability in the striatum could be due to pre-synaptic mechanisms, such as altered glutamate release properties. Post-synaptic changes in MSN excitability may result from changes in spine density, synapse number per spine, the number or subunit composition of AMPARs at synapses, or alterations in intrinsic membrane properties (Arbuthnott 1974; Cepeda 1993; Day et al. 2006; Hernandez-Lopez et al. 2000; Nisenbaum 1986; Orr et al. 1986; Schultz and Ungerstedt 1978; Tseng et al. 2001).

While we cannot exclude an exercise related effect on AMPAR number, our studies support that the decrease in sEPSC amplitude observed after exercise may be due to an increase in GluR2-containing AMPARs. Subunit composition itself is a major determinant for AMPAR conductance properties. AMPARs containing GluR2 with either GluR1 or GluR3 are characterized by Ca^{2+} impermeability and low channel conductance. These changes result from post-transcriptional modification of the GluR2 transcript whereby a glutamine (Q) codon is converted to a codon for arginine (R), which resides within the channel pore and due to its positive charge moiety impedes cation flow (Pellegrini-Giampietro et al. 1997; Swanson 1997). We examined the relative abundance of GluR2 mRNA transcripts encoding either the Q or R codon. Using a RT-PCR based approach in conjunction with site-specific restriction enzyme analysis, we observed no changes in the levels of Q encoding GluR2 transcript between any groups (data not shown), thus indicating that GluR2-containing AMPARs expressed in our exercise study are low conductance channels (Takuma et al. 1999).

An alternative explanation for differences observed in sEPSC amplitude between MPTP and MPTP plus exercise mice may be due to alterations in presynaptic glutamate release of corticostriatal glutamatergic terminals. However, analysis of pair pulse ratio (PPR), an indicator of glutamate availability, did not show any significant change between treatment groups. In

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addition, we did not observe any difference in sEPSC frequency, indicative of similar numbers of presynaptic terminals. This is in contrast to the robust and consistent increase in sEPSC frequency, with an accompanied increase in pre-synaptic glutamate release observed in 6-OHDA lesioned rats examined at similar post-lesion time points (Centonze et al. 2005; Gubellini et al. 2002; Picconi et al. 2002; Tang et al. 2000). A key difference between the 6-OHDA and MPTP models is the degree of dopamine loss. The MPTP-lesioning regimen used in these studies leads to a 78% depletion in striatal dopamine at completion of the exercise paradigm (42 days post-lesioning), where by contrast, most 6-OHDA studies used to examine striatal sEPSC's report dopamine depletion in excess of 90% or greater (Centonze et al. 2005; Gubellini et al. 2002; Jakowec et al. 2004; Petzinger et al. 2007; Picconi et al. 2002).

Using whole-cell voltage clamp and stimulation of glutamatergic afferents, we also observed a decrease in the input-output ratio of evoked EPSCs of MSNs from MPTP plus exercise compared with MPTP mice. Plasticity at excitatory synapses involves alterations in AMPAR subunit composition and trafficking at synaptic membranes (Davies et al. 2008). Accordingly a reduction in the amplitude of evoked EPSCs and sEPSCs, is consistent with either (i) an increase in GluR2 containing channels (MPTP animals) and/or an (ii) increase in GluR2880 expression (MPTP and saline animals) with increase in AMPAR trafficking as seen in exercised animals. Together these findings support the role that intensive exercise may play in modulating synaptic strength. Exercise also led to alterations in the splice variant expression of both GluR1 and GluR2. Treadmill exercise decreased the expression of flip splice variant of both GluR1 and GluR2, suggesting that high intensity treadmill exercise may also lead to a decrease in synaptic strength via changes in AMPAR kinetics of the post-synaptic glutamatergic response. Studies are ongoing to further characterize the kinetics of the AMPAR response in exercised animals.

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While the mechanism is not entirely clear, evoked EPSCs were actually larger in the saline group than in the MPTP group. It is possible that these differences may be explained, in part, by a pathological decrease in dendritic spine density and associated loss of cortical synapses. Previous studies reported that, following MPTP administration, striatal MSNs experience a decrease in spine density, which may thus lead to a consequent reduction in AMPAR number (Day et al. 2006; Ingham et al. 1989; Neely et al. 2007). Our findings are consistent with the proposed hypothesis that while dopamine depletion may cause a total decrease in dendritic spine density, remaining synapses become hyperexcitable, leading to abnormal synaptic drive in surviving synapses (Day et al. 2006). Our study also indicates that the increase in synaptic excitability may be explained in part by increased expression of GluR1-containing AMPARs at remaining synapses and that exercise may reverse this excitability state through increasing the synaptic expression of GluR2-containing AMPARs.

In conclusion, reduced AMPAR conductance through increased expression of GluR2 and its phosphorylated state may contribute to the exercise-induced improvement in motor performance in MPTP mice. There is compelling evidence in the literature that the loss of nigral dopaminergic neurons is responsible for increased corticostriatal glutamatergic drive at the level of striatal MSNs, contributing to the motor deficits in PD (Cepeda et al. 2001; Meshul et al. 1999; Neely et al. 2007; Schwarting and Huston 1996). One potential mechanism by which exercise may drive experience-dependent neuroplasticity in PD and lead to behavioral benefit is through mitigating corticostriatal hyperactivity by diminishing AMPAR-mediated conductance. In accordance with this finding, AMPAR antagonists have been demonstrated to help alleviate motor symptoms in PD (Chase et al. 2000; Klockgether et al. 1991). Given the roles AMPAR trafficking and subunit composition in synaptic plasticity, our study provides evidence for AMPARs playing a role in experience-dependent plasticity of the basal ganglia in both the healthy and injured (dopamine depleted) basal ganglia. Findings from our study may also

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explain the behavioral benefits of exercise in Parkinson's disease and help identify a potential disease modifying mechanism through intense motor practice.

Acknowledgements: The first two authors contributed equally to this manuscript. Special thanks to Elizabeth Hogg and Avery Abernathy for their technical expertise. A special thanks to Friends of the USC Parkinson's Disease Research Group including George and Marylou Boone and Walter and Susan Doniger. This paper is dedicated in memory of George Boone, for his constant encouragement and support of neuroscience research.

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Legends for Tables and Figures

Table 1: HPLC Analysis of striatal dopamine and its metabolites. The concentration of dopamine, DOPAC, and HVA and turnover ratio were analyzed in each experimental group (n = 6 per group) at 10 d post-lesioning and 42 d post lesioning (corresponding to 28 d post-exercise). The turnover ratio is defined as [(DOPAC + HVA)/dopamine]. At day 10 post-lesioning there was a significant effect of MPTP on dopamine, DOPAC levels, and turnover ratio, compared with saline animals (* represents significance at $p < 0.05$). At 42 days post lesioning (28 days post-exercise) there was also a significant effect of MPTP on dopamine, DOPAC and HVA levels compared with saline-treated animals (* represents significance at $P< 0.05$). There was no significant effect of exercise on the MPTP-lesioned group. There was a significant effect of exercise on the saline treated group, where saline plus exercise mice had a higher level of striatal dopamine compared to saline mice plus no exercise (+ represents significance at $p < 0.01$).

Figure 1: Comparison of the treadmill running velocity of MPTP and saline mice. Saline and MPTP-lesioned mice were run on the motorized treadmill for 28 d (5 d per week). The running velocity of each group of mice (n = 12) per groups were determined three times per week and compared. MPTP-lesioned mice were significantly slower in velocity compared to saline animals on weeks 1-4 (The asterisk represents significant differences between the two groups at $p < 0.05$). Both MPTP-lesioned and saline groups improved in running velocity. There were no significant differences in running velocity between MPTP-lesioned and saline animals at weeks 5 and 6. Graphs represent average running velocity over three days for each group. Error bars indicate SEM.

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Figure 2: Analysis of GluR2 protein expression after exercise. The relative expression of GluR2 protein was determined using immunohistochemistry and computer assisted analysis followed by cell counting after 28 d of exercise. Sections at the level of the mid-striatum were immunostained for GluR2. **(A)** The upper panel shows low-magnification (40x) and the lower panels high-magnification (100x) images of coronal sections at a region corresponding to the dorsolateral striatum (mid-striatum) of representative animals. Scale bar is 200 microns for upper panels, and 40 microns for lower panels. The corpus callosum is represented by CC. **(B)** There was a significant increase in the total number of GluR2 positive neurons in the dorsolateral striatum of the MPTP-lesioned mouse compared to saline animals. This increase was due to a significant increase in the total number of GluR2 positive neurons in the dorsolateral striatum of MPTP + exercise animals. **(C)** There were no significant differences in the optical density of neuropil staining of the dorsolateral striatum between any of the treatment groups. Results indicate mean ± SEM. (asterisk represents significance at $p < 0.01$)

Figure 3: Western immunoblotting analysis of dorsolateral striatal expression of GluR1 and GluR2 protein expression in Synaptoneurosomes. The upper panels show representative scans of western immunoblotting results for GluR2 and GluR1 with an antibody against alpha-tubulin in the lower bands to normalize for gel loading. The lower panels show graphical depiction of data from 3 independent experiments showing (Figure 3A) a significant increase in GluR2 protein in the MPTP plus exercise group, and (Figure 3B) no significant changes in GluR1 protein in any treatment group. Results display mean ± SEM. Asterisk represents significance at $p < 0.05$.

Figure 4: Analysis of Phosphorylated GluR2-Serine880 protein expression after exercise. The relative expression of phosphorylated GluR2-Serine880 protein was determined using immunohistochemistry and computer assisted analysis followed by cell counting after 28 d of exercise. Sections at the level of the mid-striatum were immunostained for phosphorylated

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GluR2-Serine880. **(A)** The upper panel shows low-magnification (20x) and the lower panels high-magnification (100x) images of coronal sections at a region corresponding to the dorsolateral striatum (mid-striatum) of representative animals. Scale bar is 100 microns for upper panels, and 40 microns for lower panels. The corpus callosum is represented by CC. **(B)** Exercise caused a significant increase in the total number of immuno-positive cell bodies in the dorsolateral striatum of compared with non-exercised mice. There is also a significant interaction between exercise and treatment due to a greater increase in phosphorylated GluR2-Serine880 in the saline group after exercise ($F(3,21) = 5.805$; $p < 0.043$). **(C)** Analysis of phosphorylated GluR2-Serine880 immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences between the exercise and non-exercise groups ($F = 0.461$; $p = 0.516$), Error bars indicate SEM. The symbol * indicates an exercise effect and the symbol # indicates an interaction between saline and exercise. Both symbols represent significance at $p < 0.01$

Figure 5: Analysis of GluR1 protein. The relative expression of GluR1 protein was determined using immunohistochemistry and computer assisted analysis followed by cell counting after 28 d of exercise. **(A)** The upper panel shows low-magnification (20x) and the lower panels high-magnification (100x) images of coronal sections at a region corresponding to the dorsolateral striatum (mid-striatum) of representative animals. Scale bar is 200 microns for upper panels, and 40 microns for lower panels. The corpus callosum is represented by CC. **(B)** There was a slight but not significant decrease in the total number of GluR1 positive neurons in the dorsolateral striatum of the MPTP-lesioned mouse compared to saline animals. There was no significant effect of exercise in the total number of GluR1 positive neurons in either MPTP-lesioned or saline animals. **(C)** There were no significant differences in the optical density of neuropil staining of the dorsolateral striatum between any of the treatment groups. Error bars indicate SEM.

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Figure 6: Analysis of phosphorylated GluR1-Serine845 protein. The relative expression of phosphorylated GluR1-Serine845 protein after 28 d of exercise was determined using immunohistochemistry. **(A)** The upper panel shows low-magnification (40x) and the lower panels high-magnification (100x) images of coronal sections at a region corresponding to the dorsolateral striatum (mid-striatum). Scale bar is 100 microns for upper panels and 40 microns for lower panels. The corpus callosum is represented by CC. **(B)** There were no significant differences in the number of immuno-positive cells between MPTP and saline animals, nor between exercise and non-exercised animals in the dorsolateral striatum. **(C)** Analysis of phosphorylated GluR1-Serine845 immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences between MPTP and saline animals and exercise and non-exercised groups. Error bars indicate SEM.

Figure 7: In situ hybridization histochemical analysis of GluR1 and GluR2 mRNA transcript expression in dorsolateral striatum. The upper left panel shows representative images from the dorsolateral striatum of emulsion-dipped sections captured at 40X magnification for groups (A) Saline, (B) Saline+exercise, (C) MPTP, and (D) MPTP+exercise. The graph in the lower left depicts the number of grains per cell area (grains/square micron) indicating reduced GluR1 expression in saline+exercise mice only. The panel in the upper right shows representative images from the dorsolateral striatum of emulsion-dipped sections captured at 40X magnification for groups (E) Saline, (F) Saline+exercise, (G) MPTP, and (H) MPTP+exercise. The graph in the lower right depicts the number of grains per cell area (grains/square micron) indicating elevated expression of GluR2 transcripts in the MPTP and MPTP+exercise groups. Both graphs show data as mean ± SEM. The scale bar in panel H represents 100 microns and applies to all panels.

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Figure 8. qRT-PCR Analysis of GluR2 and GluR1 transcript splice variant expression. (A) There was a significant decrease in the expression of GluR2-flip in exercise compared to non-exercise mice. There was also a significant decrease in MPTP-lesioned compared to saline animals on GluR2-flip expression. **(B)** There was no significant effect of exercise on GluR2-flop expression ($F(3,12) = 0.04$; $p = 0.843$). There was a significant increase in GluR2-flop expression in MPTP-lesioned mice compared to saline. **(C)** There was a significant decrease in the expression of GluR1-flip in exercise compared to non-exercise mice. There was no significant effect of MPTP on GluR1-flip expression. **(D)** There was no significant effect of exercise on GluR1-flop splice variant ($F(3,12) = 3.646$; $p = 0.093$). There was a significant decrease in GluR1-flop expression in MPTP-lesioned mice compared to saline. The symbols "*" compares exercise to non-exercise and "#" compared saline to MPTP and both represent significance at $p < 0.001$.

Figure 9. Exercise reduces inward rectification of AMPA receptor mediated corticostriatal EPSCs in MPTP treated mice. (A) Example of EPSCs evoked in dorsolateral striatal neurons at holding potentials of –80 and +60 mV from MPTP alone and MPTP + exercise mice (stimulus intensity = 80 μA). **(B)** Current-voltage plots of synaptic currents evoked for the cells illustrated in A. Synaptic currents were normalized to the peak synaptic current evoked at –80 mV for ease of presentation. **(C)** Measurement of rectification in current-voltage relationship, or the rectification index (RI)(ratio of synaptic conductance at +60 mV vs –80 mV). Synapses from MPTP mice demonstrated a significantly lower RI (more rectification) versus MPTP plus Exercise mice or saline mice ($p < 0.03$).

Figure 10. Exercise reverses the increase in spontaneous excitatory post-synaptic current (sEPSC) amplitude created by MPTP. (A) Representative 10 second-long recordings illustrating sEPSCs from mouse medium spiny neurons in each experimental group. **(B and C)** Cumulative frequency histograms of sEPSC amplitude and inter-event intervals. A significant

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increase of events amplitude could be seen in neurons from MPTP mice only. **(B)** There were no significant differences observed in events frequency between any of the groups **(C).**

Figure 11: Exercise reduces the input-output relationship for corticostriatal synapse in MPTP treated mice. **(A)** Example of EPSCs evoked from dorsolateral corticostriatal synapses in brain slices taken from MPTP and MPTP plus exercise mice. EPSCs were produced by the stimulation intensities shown in the x-axis of B. EPSCs were generated at a holding potential of –80 mV for all input-output experiments. **(B)** Plot of input (stimulation intensity) – output (EPSC amplitude) for corticostriatal synapse from saline, MPTP and MPTP plus exercise mice. **(C)** Bar graph of slope calculated for the input-output relationship for corticostriatal synapses as shown in B Exercise significantly reduced the slope of this relationship in MPTP mice (* $p<0.03$) The slope of the input-output relationship for the saline group was significantly higher than either of the MPTP groups (+ $p < 0.01$)

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Table 1:

			Dopamine	DOPAC	HVA	Turnover
Day 10 Post-MPTP	No Exercise	Saline	269.5 ± 24.9	33.4 ± 4.5	33.1 ± 3.2	0.3 ± 0.08
		MPTP	48.0 ± 8.4*	11.7 ± 1.9*	87.2 ± 9.6	2.3 ± 0.28*
Day 42 Post-MPTP	No Exercise	Saline	246.9 ± 19.8	36.5 ± 4.5	27.4 ± 1.7	0.3 ± 0.01
		MPTP	77.9 ± 12.0*	14.7 ± 2.4*	13.5 ± 1.9*	0.4 ± 0.01
	Exercise (28 days)	Saline	315.2 ± 9.0#	39.3 ± 2.8	25.9 ± 1.3	0.2 ± 0.01
		MPTP	69.8 ± 11.7*	11.3 ± 1.5*	13.5 ± 1.2*	0.4 ± 0.05

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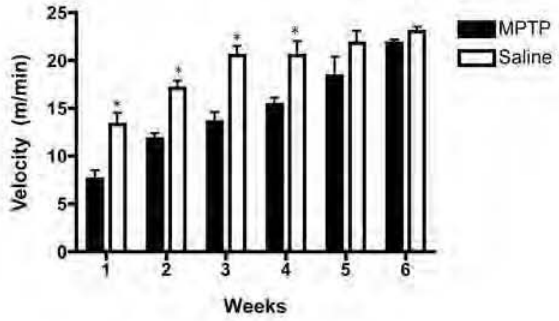

Figure 1
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Figure 2: GluR2

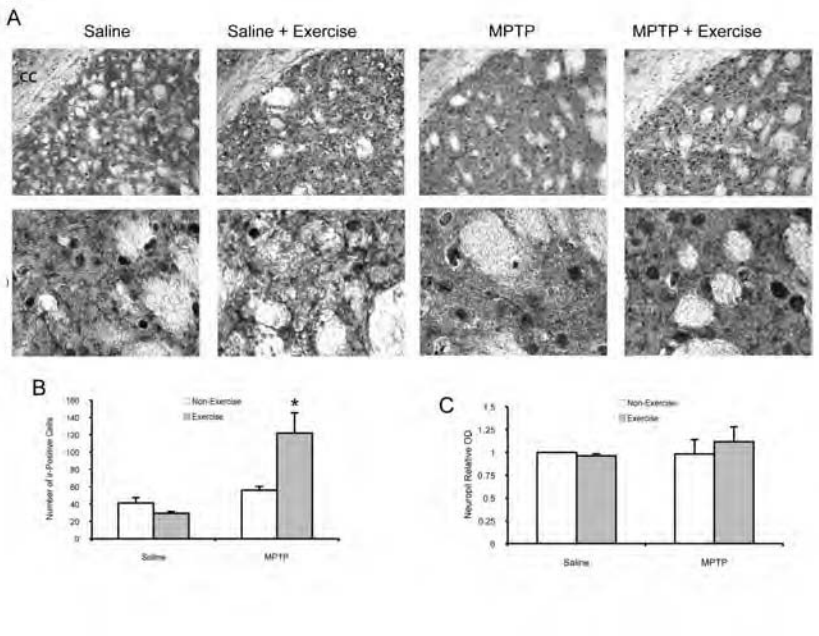

Figure 2
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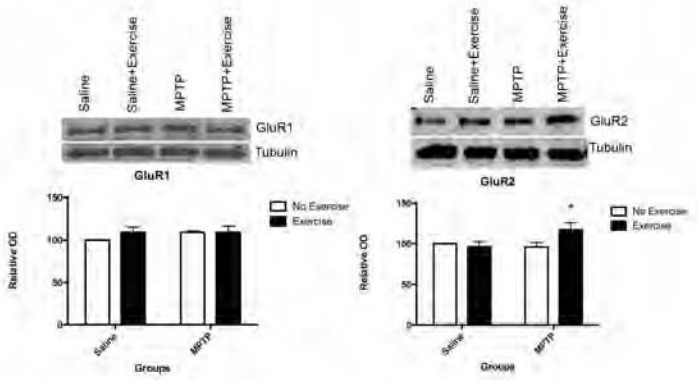
Figure 3: GluR1 and GluR2 western blot



GluR2/1 WIB
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Figure 4: GluR2-Ser880

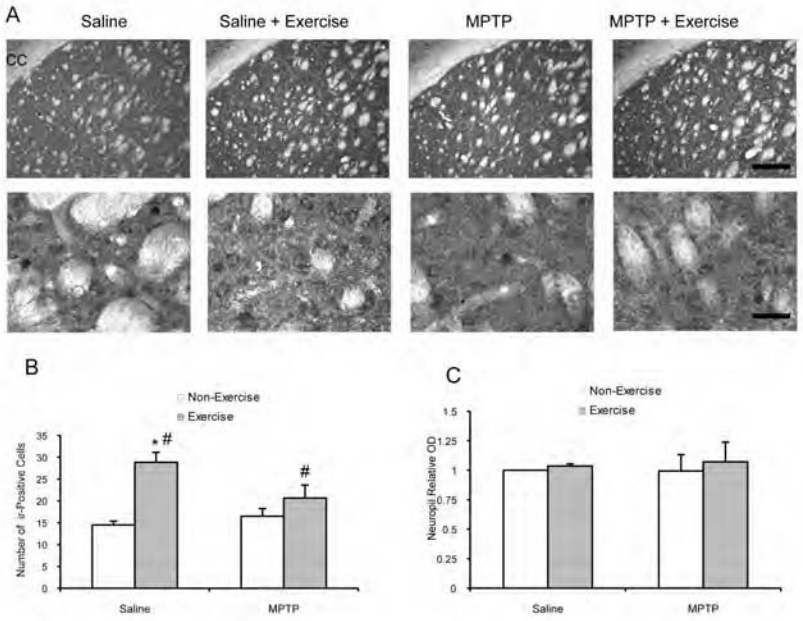

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Figure 5: GluR1

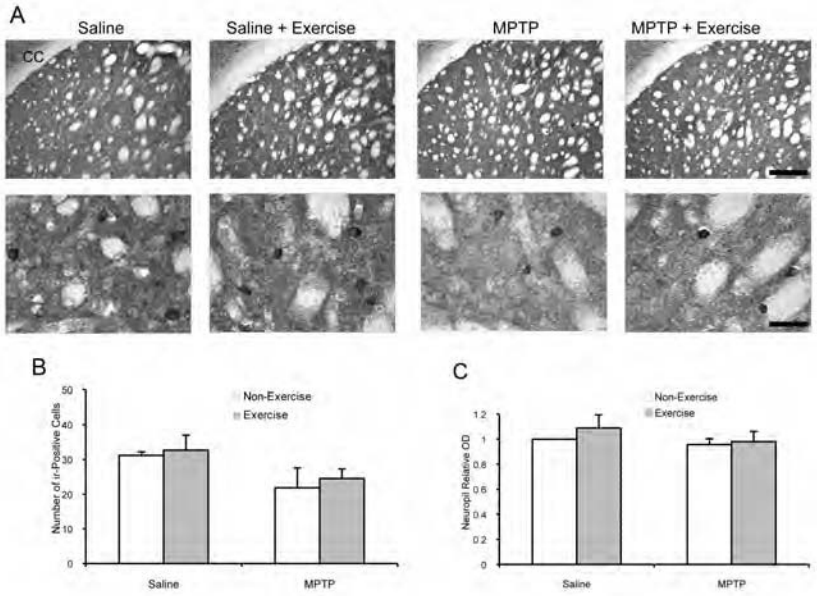

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Figure 6: GluR1 S845

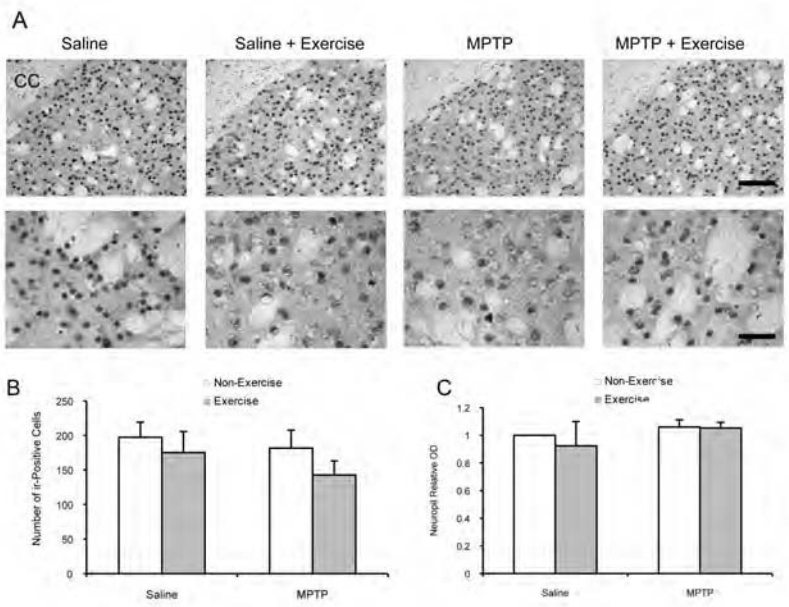

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Figure 7: GluR1 an GluR2 ISH

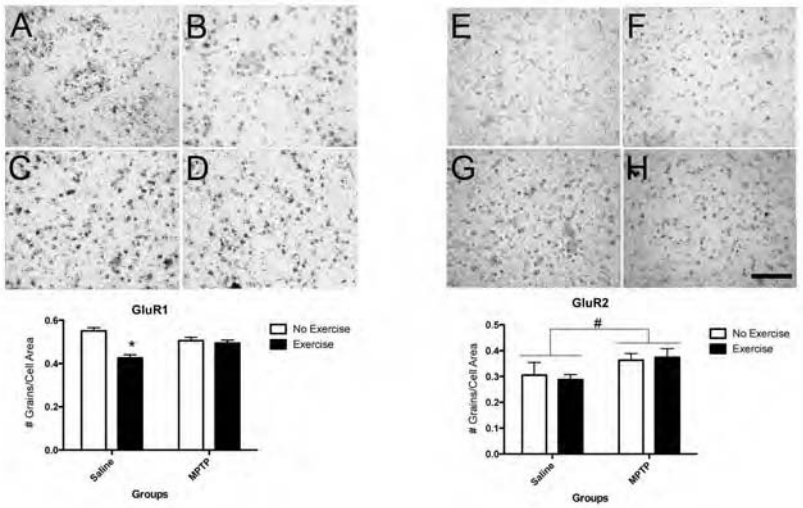

Figure 7
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Figure 8: qRT-PCR

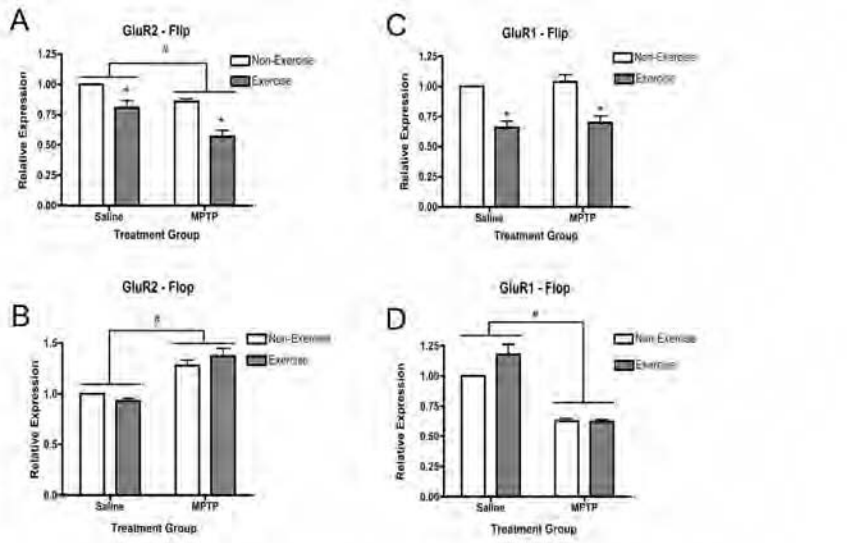

Figure 8
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Figure 9

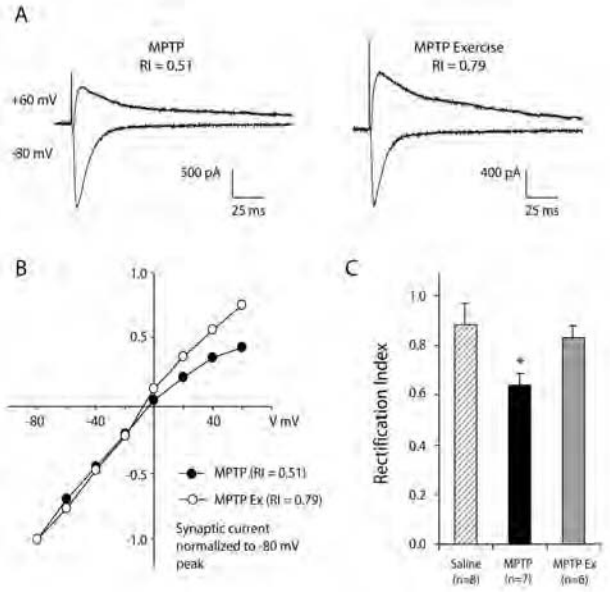

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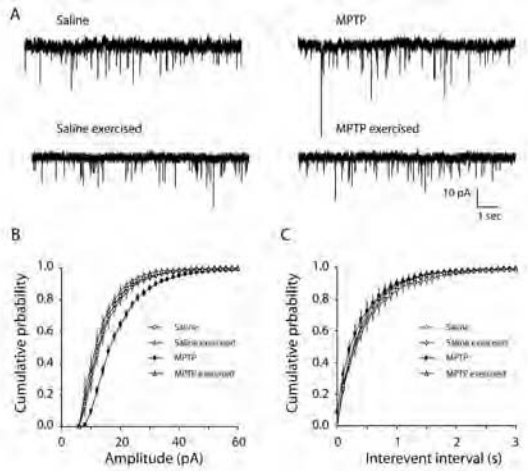
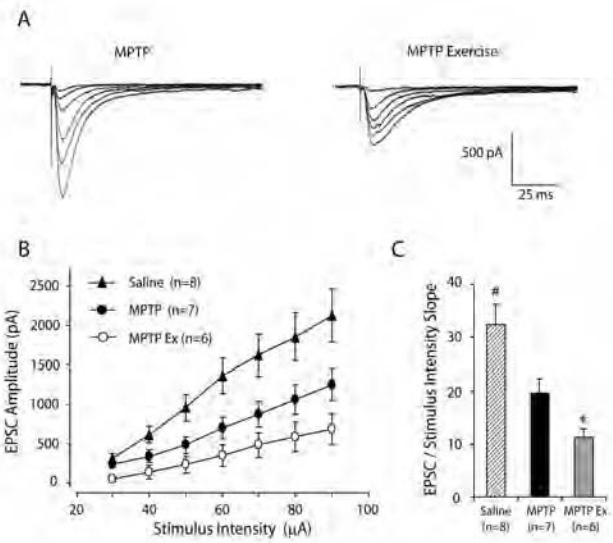
Figure 10: sEPSC



sEPSC
203x254mm (300 x 300 DPI)

Figure 11



input/output
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